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Gas chromatographic analysis of fatty acids from dialyzed lipoproteins*

ALEX V. NICHOLS, CARL S. REHNBORG, and FRANK T. LINDGREN

Donner Laboratory of Biophysics and Medical Physics, Lawrence Radiation Laboratory, University of California, Berkeley 4, California

[Received for publication December 14, 1960]

SUMMARY

Dialysis of three major lipoprotein fractions from human serum, S_f20-10^5, S_f0-20, and -$S_{1.20}$0-16, against low concentrations of cupric ion is associated with accelerated chemical alteration of polyunsaturated fatty acids and cholesterol. Less extensive changes in these acids were observed during dialysis against ion-free water. Reaction products of these chemical alterations were irreversibly retained on succinic acid-diethylene glycol polyester gas chromatographic columns. Present evidence suggests that these reaction products are relatively polar compounds, probably in the chemical class of lipoperoxides.

During dialysis of serum lipoproteins against cupric ion, Ray *et al.* (1) observed marked changes in analytic ultracentrifugal patterns. Their studies established cupric ion as a catalyst for reactions leading to lipoprotein degradation. They suggested that this reaction was oxidative in nature and that unsaturated compounds, such as unsaturated fatty acids, were involved. Degradative effects have also been observed during isolation and storage of lipoprotein fractions (2). Gurd (3) has associated such degradative or aging effects in lipoprotein solutions with shifts in ultraviolet absorption spectra which are indicative of lipoperoxide formation.

Gas-liquid chromatographic techniques have provided a sensitive method for the determination of fatty acids. In this report we have applied these techniques to the measurement of fatty acids from lipoprotein fractions exposed to different conditions of dialysis. It was of special interest to establish the fatty acids involved in the cupric ion reaction, and their elution properties on a succinic acid-diethylene glycol polyester chromatographic column before and after dialysis. The relationship of the fatty acid changes to the gross macromolecular degradation is discussed.

* This investigation was supported by the United States Atomic Energy Commission, Washington, D. C.

METHODS

Ultracentrifugal Isolation and Analysis of Lipoprotein Fractions. Ultracentrifugal flotation techniques, reported previously (4), were used for the isolation and analysis of three lipoprotein fractions from human serum. The specific lipoprotein fractions isolated were the S_f20-10^5, S_f0-20, and -$S_{1.20}$0-16. These ultracentrifugal designations and their relationship to the physical properties of these lipoprotein fractions have been reported in previous communications (5). Two individual sera were used in this investigation. Serum from a nonfasting 52-year-old male was used for the isolation of S_f20-10^5 lipoproteins. The S_f0-20 and -$S_{1.20}$0-16 lipoproteins were isolated from a nonfasting 22-year-old female. No precautions against the presence of metal ions were taken during the ultracentrifugal isolation procedures. Upon isolation, the lipoprotein fractions were immediately subjected to dialysis.

Dialysis Procedures. Lipoprotein fractions were dialyzed in Visking cellulose casing ($^{18}/_{32}$-inch diameter). The tubing previously was washed and tested with ion-free water. Ion-free water was prepared by passing laboratory distilled water through the following large capacity, ion-exchange columns: first, Dowex-50, then Dowex-1, and finally an equal part mixture of Dowex-50 and Dowex-1. Water prepared by this procedure was considered ion-free and was used in the preparation of cupric ion solutions for dialysis.

The lipoprotein fractions were dialyzed against ion-free water and ion-free water containing approximately 1.5×10^{-5} moles/liter of cupric ion. In the dialysis procedure 1 ml of lipoprotein solution was dialyzed against approximately 200 ml of solution. A small amount of air was present in the dialysis bag. Dialysis was carried out on a gently rocking platform in a refrigerator maintained between 0° and 4°. Dialysis solutions were changed approximately every 12 hours. Upon sampling, the lipoprotein fractions were carefully removed from the dialysis tubing, the tubing was washed with a small amount of ion-free water, and this wash was pooled together with the lipoprotein solution for further extraction procedures.

The schedule for sampling of the S_f20-10^5 lipoprotein fraction was as follows: 0, 25.5, 29.25, 44.0, and 138.75 hours after beginning of dialysis. Both S_f0-20 and -$S_{1.20}$0-16 fractions were sampled at 0, 11.0, 39.25, 56.5, and 128.75 hours after dialysis. The physical appearance of the lipoprotein fractions was noted throughout the course of the dialysis.

Lipid Procedures. Lipids from control and dialyzed lipoprotein fractions were extracted by procedures based on the method of Sperry and Brand (6). Lipid extracts were methylated by transesterification with methanol according to the method of Stoffel *et al.* (7). Cholesterol present in the lipid extract was not removed from the fatty acid methyl esters prior to their injection into the chromatographic column.

Gas-Liquid Chromatographic Procedures. Gas-liquid chromatographic analyses were performed on a 52-inch column (6 mm i.d.) unit reported by Upham *et al.* (8). Analyses were made at 195°, utilizing a strontium-90 ionization detection system (9, 10). Packing material consisted of Chromosorb (48 to 65 mesh) coated with 30% (w/w) succinic acid-diethylene glycol polyester (LAC-2R-728®).[1] Argon was used as the carrier gas. Peak heights and elution time values were tabulated for every fatty acid ester peak on the chromatograms. This information was put on punched cards and the calculation of fatty acid composition was performed by computer (11). The known major fatty acids are reported according to the nomenclature proposed by Dole *et al.* (12). Minor components have been grouped into four separate classes. The classification of these components is based on their elution times relative to known methyl esters under the above conditions. Thus these four classes have been designated: class A, methyl esters eluting before 16:0 (methyl palmitate); class B, methyl esters eluting between 16:0 and 18:0 (methyl stearate); class C, methyl esters eluting between 18:2 (methyl linoleate) and 20:4 (methyl arachidonate); and class D, methyl esters eluting after 20:4. In this report the identified components are: 16:0 (methyl palmitate), 16:1 (methyl palmitoleate); 18:0 (methyl stearate); 18:1 (methyl oleate and methyl elaidate), 18:2 (methyl linoleate), and 20:4 (methyl arachidonate). A reference chromatogram indicating the above classification system is presented in Figure 1.

[1] Cambridge Industries Company, Inc., 101 Potter Street, Cambridge 42, Mass.

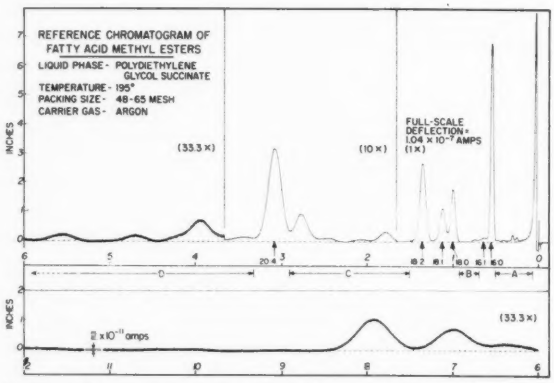

FIG. 1. Reference gas-liquid chromatogram of fatty acid methyl esters showing elution properties of known major components. Relatively minor components are grouped into four classes, *A*, *B*, *C*, and *D*, as indicated on the elution time scale. The elution time scale is marked off in units of methyl stearate elution times.

RESULTS AND DISCUSSION

Physical Changes in Lipoproteins During Dialysis. After 25.5 hours of dialysis against ion-free water, lipoproteins of the S_f20-10^5 fraction showed only a very slight turbidity. There was no further detectable aggregation or color change during the dialysis. Upon dialysis against cupric ion, there was a slight increase in turbidity and loss of color (yellow to white) after 25.5 hours. At approximately 29.25 hours a gross turbidity with marked aggregation was observed. The turbidity and loss of color persisted to termination of sampling.

Dialysis of the S_f0-20 lipoprotein fraction resulted in slight turbidity and color change at 56.5 hours, followed by marked aggregation at approximately 69.5 hours. Upon dialysis against cupric ion, gross turbidity and loss of color were observed at approximately 11 hours. Considerable aggregation and color loss were observed throughout the remainder of the experiment.

During dialysis against ion-free water, the -$S_{1.20}$0-16 lipoprotein solution remained clear and showed little color change throughout the sampling period. Upon

dialysis against cupric ion, slight turbidity and color loss were observed within 11 hours. Marked turbidity was noted around 39.25 hours. Turbidity and color changes persisted to the end of the experiment.

The above observations show a definite similarity between the S_f20-10^5 and $-S_{1.20}0$-16 lipoproteins in their responses to cupric ion and ion-free water dialysis. However, the physical changes in S_f0-20 lipoprotein solutions occurred sooner during cupric ion dialysis than was observed with the other lipoprotein fractions. Moreover, dialysis of S_f0-20 lipoproteins against ion-free water led to gross physical changes absent in the other fractions under the same conditions. These observations suggest that the S_f0-20 lipoproteins are more sensitive to dialysis procedures.

Analytic Ultracentrifugal Results. Appropriate analytic ultracentrifugal analyses were performed on the S_f20-10^5, S_f0-20, and $-S_{1.20}0$-16 lipoprotein fractions at 47.5, 40.25, and 40.25 hours, respectively, after cupric ion dialysis. At these times all fractions were markedly turbid, and the ultracentrifugal patterns showed no detectable flotation of intact lipoproteins. The aggregated material was rapidly packed at the meniscus during the acceleration of the ultracentrifuge to full speed (52,640 rpm). It is thus apparent that the cupric ion dialysis leads to gross lipoprotein aggregation, which is not reversed by suspension in aqueous solutions of sodium bromide. In this experiment no sedimenting material was noted in the lipoprotein solutions upon analytic ultracentrifugation.

Gas-Liquid Chromatographic Analyses. The results of the gas-liquid chromatographic analyses are presented in Tables 1 to 3. Fatty acid methyl esters are tabulated for lipoprotein fractions exposed to the following conditions: (*a*) no dialysis, (*b*) dialysis against ion-free water, and (*c*) dialysis against cupric ion solution 1.5×10^{-5} moles/liter. The content of the various fatty acid methyl esters is expressed as weight per cent of the total methyl esters injected onto the column.

TABLE 1. PERCENTAGE FATTY ACID COMPOSITION OF S_f20-10^5 LIPOPROTEIN FRACTION AS A FUNCTION OF CONDITIONS AND DURATION OF DIALYSIS

Fatty Acids	No Dialysis* 0 hrs.	No Dialysis* 138.75 hrs.	Dialysis Against Ion-Free Water 25.5 hrs.	Dialysis Against Ion-Free Water 138.75 hrs.	Dialysis Against 1.5×10^{-5} Moles/liter Cu++ 25.5 hrs.	29.25 hrs.	44.0 hrs.	138.75 hrs.
Class A	4.0	3.5	3.8	3.0	3.6	2.8	3.1	4.4
16:0	25.7	26.6	25.6	24.1	24.4	23.0	24.4	24.5
16:1	6.8	7.1	7.1	6.2	6.5	6.0	5.9	5.8
Class B	0.5	0.5	0.5	0.6	0.6	0.5	0.5	1.1
18:0	6.4	6.3	6.1	6.2	6.0	5.7	5.9	6.0
18:1	30.6	31.4	29.9	29.2	29.3	27.5	27.7	25.2
18:2	18.3	18.8	18.8	16.2	15.3	14.1	9.6	3.0
Class C	4.7	3.5	3.6	3.3	3.3	2.5	2.4	2.4
20:4	2.9	2.8	2.8	2.2	1.3	1.0	0.6	0.4
Retained†	0	0	2.2	9.3	9.5	17.2	20.0	27.5

* Nondialyzed control samples were stored at 0°–4° prior to extraction.

† Altered fatty acids irreversibly retained on column. (Values were estimated from a comparison of the injected weight of methylated lipid sample with the amount detected on its gas-liquid chromatogram. It was assumed that in the control samples all of the injected methyl esters were eluted.)

TABLE 2. PERCENTAGE FATTY ACID COMPOSITION OF S_f0-20 LIPOPROTEIN FRACTION AS A FUNCTION OF CONDITIONS AND DURATION OF DIALYSIS

Fatty Acids	No Dialysis 0 hrs.	Dialysis Against Ion-Free Water 11 hrs.	Dialysis Against Ion-Free Water 128.75 hrs.	Dialysis Against 1.5×10^{-5} Moles/liter Cu++ 11 hrs.	56.5 hrs.	128.75 hrs.
Class A	1.4	1.2	1.5	1.3	4.0	3.1
16:0	18.6	15.7	17.3	16.1	21.5	17.1
16:1	3.2	2.8	3.1	2.8	3.7	1.9
Class B	1.1	0.9	1.1	1.0	2.9	2.7
18:0	6.1	5.0	5.4	5.1	6.8	5.6
18:1	21.5	19.9	19.6	19.4	18.5	7.0
18:2	36.9	30.8	29.8	17.9	2.1	0.5
Class C	3.0	2.4	2.5	1.7	2.3	2.6
20:4	6.2	4.7	3.2	1.3		
Class D	2.2	1.4	1.2	0.8	1.7	1.2
Retained*	0	15.3	15.3	32.8	36.5	58.3

* Altered fatty acids irreversibly retained on column. (Values were estimated from a comparison of the injected weight of methylated lipid sample with the amount detected on its gas-liquid chromatogram. It was assumed that in the control samples all of the injected methyl esters were eluted.)

TABLE 3. PERCENTAGE FATTY ACID COMPOSITION OF $-S_{1.20}0$-16 LIPOPROTEIN FRACTION AS A FUNCTION OF CONDITIONS AND DURATION OF DIALYSIS

Fatty Acids	No Dialysis 0 hrs.	Dialysis Against Ion-Free Water 11 hrs.	Dialysis Against Ion-Free Water 128.75 hrs.	Dialysis Against 1.5×10^{-5} Moles/liter Cu++ 11 hrs.	39.25 hrs.	56.5 hrs.	128.75 hrs.
Class A	1.7	1.7	1.5	1.4	2.4	1.8	1.5
16:0	20.6	19.8	16.9	17.6	21.1	17.8	18.9
16:1	2.8	2.7	2.4	2.6	2.7	2.5	2.0
Class B	1.2	0.8	1.0	1.1	2.3	2.2	1.7
18:0	8.3	7.8	6.9	7.8	7.9	6.9	7.8
18:1	17.8	18.3	14.8	15.8	13.8	11.0	11.4
18:2	33.9	33.6	25.5	26.0	1.7	1.0	0.7
Class C	4.4	3.4	2.5	2.1	1.3	1.5	1.7
20:4	9.4	9.3	5.3	5.2			
Class D		2.0	1.5	0.7	1.1	1.0	1.1
Retained*	0	0.8	21.7	20.0	45.6	54.2	53.3

* Altered fatty acids irreversibly retained on column. (Values were estimated from a comparison of the injected weight of methylated lipid sample with the amount detected on its gas-liquid chromatogram. It was assumed that in the control samples all of the injected methyl esters were eluted.)

Significant changes occur in the fatty acid composition of each lipoprotein fraction after dialysis against cupric ion. Dialysis against ion-free water is associated with some alterations in composition, but considerably less than are observed with cupric ion. In particular, during dialysis against cupric ion, significant decreases in the polyunsaturated fatty acids, linoleate and arachi-

donate, are observed. The content of oleic acid is reduced in all fractions and shows a pronounced drop in the S_f0-20 lipoprotein fraction. In these gas-liquid chromatograms no new major peaks appear which might indicate the elution of chemical products arising from the alteration of the polyunsaturated fatty acids. On the contrary, there is evidence that the products of polyunsaturated fatty acid alteration are irreversibly retained on the succinic acid-diethylene glycol polyester coating. This evidence is presented at the bottom of Tables 1 to 3, where for the same weight of methylated lipid injected onto the column there is a significant reduction, especially after cupric ion dialysis, in the total fatty acid methyl esters detected with gas-liquid chromatographic analysis. Such differences are also observed for the lipoprotein fractions dialyzed against ion-free water, but to a much lesser extent than in the cupric ion dialysis.

Our present knowledge of the chemical structure and properties of the reaction products is only indirect. They are apparently retained on the relatively polar succinic acid-diethylene glycol polyester resin in a manner similar to other relatively polar organic compounds which we have studied. Thus, on this resin certain lipid and steroid compounds containing hydroxyl or ketone groups (e.g., dihydroxy stearic acid and cholesten-3-one) have exhibited either exceedingly long retention times, or were not eluted at all.[2] This similarity in elution behavior leads us to suspect that polar oxygen-containing compounds may be formed during cupric ion dialysis. Published studies on copper-catalyzed oxidation of linoleic acid indicate the formation of hydroperoxides resulting from the action of copper on peroxides present in the acid (13). If hydroperoxides are formed during lipoprotein dialysis against cupric ion, then, from the above, it would be reasonable to expect that such compounds would be retained either irreversibly or appreciably longer than known unaltered fatty acids. It is, however, entirely possible that the new compounds may not be hydroperoxides but are some other altered forms of polyunsaturated fatty acids which can be retained on the succinic acid polyester resin.

Concurrent with the observed changes in fatty acids, there occur significant reductions in the gas-liquid chromatographic peaks associated with the elution of cholesterol present in our lipid mixtures (14). It would thus appear that cholesterol is also altered during the dialysis of lipoprotein fractions, and that the resultant products are not detected by the gas-liquid chromatographic system. It is reasonable to suspect that cholesterol too may form polar products which are irreversibly retained on the polyester column.

The marked aggregation of the lipoprotein fractions upon cupric ion dialysis implies an alteration in the surface properties of the lipoprotein macromolecules associated with chemical changes in polyunsaturated fatty acids and cholesterol. A similar phenomenon, under oxidative conditions, has been observed for electron microscopy (15) during the fixation of lipoprotein molecules by acid solutions of osmium tetroxide. In this case the lipoprotein aggregates are redispersed by adjusting the pH of the solution to approximately 8. The ability to redisperse these aggregates and the general agreement between the resulting electron micrograph data on lipoprotein size and shape, and ultracentrifugal or light scattering measurements for the same variables suggest that oxidative changes primarily alter the surface properties of lipoproteins without significantly disrupting their macromolecular structure. This may also be the case with lipoprotein molecules after dialysis against cupric ion. The influence of pH and ionic strength on the lipoprotein aggregates from cupric ion dialysis will be reported.

Recent investigations in animals on the pharmacologic action of fatty acid oxidation products indicate considerable alterations in metabolism and physiologic status of animals after administration of these products (16). The incorporation of these products into lipoprotein structures may so alter the surface properties, as was noted above, that the usual metabolic reactions involving lipoprotein macromolecules may be significantly affected. Thus it may be of considerable importance to ascertain whether in states associated with high plasma copper, or in instances of continual ingestion of oxidized lipids, there appear discernible alterations in lipoprotein stability and metabolism (17).

[2] Unpublished experiments.

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Chromatography of sphingolipids of human brain*

H. P. Schwarz, L. Dreisbach, M. Barrionuevo, A. Kleschick, and I. Kostyk

Division of Biochemistry, Philadelphia General Hospital, Philadelphia 4, Pennsylvania

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SUMMARY

Sphingolipids of the brain were prepared by solvent extraction of the tissue or from mixed brain extracts subjected to mild alkaline hydrolysis. Their components were separated on silicic-acid columns. Through use of a gradient elution with a number of chloroform-methanol mixtures and pure methanol, satisfactory fractions were obtained in an 8-hour period. The elution patterns were constant and the recoveries of hexose and alkali-stable phosphorus high. Combined chemical, infrared, and paper chromatographic techniques were used for analysis. Pure galactocerebrosides were isolated and in some instances further characterized by gas-liquid chromatography of their fatty acid derivatives. Preliminary results suggest that cerebrosides containing normal acids are eluted earlier from silicic-acid columns than those containing hydroxylated acid. A ceramide was detected in the brain from a patient with multiple sclerosis and significant amounts of glucocerebrosides in the brain of an old patient. Dihydrocerebroside-like fractions and other not characterized sphingosine derivatives were found in some instances. Pure sphingomyelins were isolated from small amounts of brain tissue.

Recent improvements of the methods for separation and analysis of lipids allow more detailed studies of sphingolipids in smaller amounts of material (1 to 4) than was possible previously. Therefore it was decided to examine the sphingolipid composition of human brain with such improved techniques. The lipids from brain specimens which showed no gross abnormalities and from brains with multiple sclerosis were examined by various methods, including chromatography and chemical and infrared analyses. The studies presented here show that in addition to regularly occurring lipids, such as galactose-containing cerebrosides and sphingomyelins, more or less uncommon sphingolipids, e.g., glucose-containing cerebrosides, ceramides, and possibly dihydrocerebrosides can be found in human brain.

EXPERIMENTAL

Material. The brain specimens were obtained as soon after death as possible.[1] Gray matter was taken from the folia of the cerebellum and the cortical gray ribbon, and white matter from the underlying cortex. Mixed gray and white matter from various parts of the brain was examined separately. The specimens were weighed and then kept frozen until lipid extractions could be carried out shortly afterward.

Preparation of Sphingolipids. The sphingolipids were prepared from brain tissue by a modification of the isolation procedure of Carter *et al.* (5). Since thus isolated sphingolipids often contained contaminating glycerophosphatides, they were purified when necessary by mild alkaline hydrolysis, which effectively destroyed the contaminants (6, 7). Purified sphingolipids were also isolated "directly" from mixed brain lipids prepared by the method of Folch *et al.* (8), and then hydrolyzed in similar manner. Nonpurified sphingolipids or mixed brain lipids containing about 3 to 15 mg alkali-stable phosphorus and 50 to 250 mg of hexose were extracted twice with hexane-acetone 1/1 (v/v), and the suspension centrifuged after each extraction. The residue was dried with a nitrogen stream, suspended in 1 N KOH, and then hydrolyzed for 24 hours at 37° with shaking. The emulsion was neutralized with 5 N HCl and then acidified to pH 1.0 with hydrochloric acid and formic acid. The sphingolipids were precipitated with acetone, washed with 2% HCl-acetone 80/20 (v/v), and with acetone, and finally dried with a stream of nitrogen.

The efficiency of the preparation of nonpurified sphingolipids by the extraction procedure of Carter *et al.* (5) was examined by determination of cerebrosides

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[1] We wish to express our gratitude to Dr. Helena E. Riggs for the supply of the material.

and sphingomyelin in the isolated material, and the residues remaining in the acetone, ether, and ethanol extracts used in that preparation. Twelve experiments carried out in this manner showed that the isolated sphingolipids contained 80% to 90% of the total cerebrosides and a somewhat lesser percentage of the total sphingomyelin found in the whole extracted material. The actual sphingolipid yields were probably only slightly lower since rather small additional amounts of cerebrosides and sphingomyelin were obtained previously, upon further extraction of a sample by Carter *et al.* (5). Purification of the isolated sphingolipids by mild alkaline hydrolysis did not cause further significant losses since up to 90% of the cerebrosides and 80% sphingomyelin were recovered after the hydrolysis. About the same yields were obtained when the purified sphingolipids were prepared "directly" from mixed brain lipids by the method already described and the intermediary step of isolation of nonpurified sphingolipids was omitted. This simplified procedure thus was applied in several instances. The results of these hydrolysis experiments are in agreement with those of Kishimoto and Radin (4) and Rapport *et al.* (9), showing that mild alkaline hydrolysis effectively destroys contaminating glycerophosphatides while leaving even labile parts of the sphingolipid structure intact. Despite these findings, however, some of the principal results obtained with hydrolyzed material were duplicated on nonhydrolyzed lipids whenever sufficient quantity of brain tissue was available for both preparations.

Column Chromatography. Fractionation of sphingolipids was carried out by adsorption chromatography on silicic-acid columns essentially as described (1, 3, 10). Twenty-five to 50 g silicic acid (Baker-Reagent, Mallinckrodt 100 mesh for chromatography or silicic acid prepared in this laboratory from sodium silicate) was activated overnight at 110°. In most instances no addition of filter-aid was required. Fresh columns were prepared in glass tubes of 24 mm diameter for each fractionation. The amount of sphingolipids applied for the chromatography was limited to about 6 mg/g silicic acid.

The lipids were dissolved in small amounts of chloroform and the somewhat turbid solutions loaded onto the columns. About 500 ml of chloroform was passed through it, thus removing contaminating cholesterol, cholesterol esters, free fatty acids, and triglycerides. The elution of sphingolipids was carried out with mixtures of chloroform-methanol of gradually increasing methanol content, but strict gradient elution (3), which may lead to overlapping of components (11), was avoided. The elution started with three 50-ml portions of chloroform-methanol 95:5, 90:10, 85:15, and 80:20 (v/v), continued with 50 ml each of 14 more mixtures, which increased in methanol content in the same progression until they reached the final composition of 5:95 (v/v), and terminated with 300 ml pure methanol. More than 20 fractionations showed that the whole procedure might be carried out easily in 8 hours, that the peaks appeared regularly in almost exactly the same parts of the chromatograms, and that about 100% of the weight, 92% to 100% of the hexose, and 83% to 89% of the phosphorus loaded onto the columns could be recovered from the eluates. Some apparently low recovery figures, particularly phosphorus recovery values, may be caused by analytical errors in the determination of numerous small fractions.

Infrared Spectroscopy. Infrared spectroscopy of sphingolipids was carried out with the modified micro KBr disk technique developed in this laboratory (1, 2). Aliquots containing 100 to 200 μg of material were suspended in 50 mg solid KBr of small particle size obtained by freeze drying. The suspensions were pressed into disks of 6 mm diameter and 1 mm thickness, and the disks were examined in the condensed beam of a Beckman IR2A infrared spectrometer furnished with a beam condenser of KBr lenses. Blank disks of plain KBr were examined with each set of determinations, and base line calculations of absorption bands were carried out in the conventional way. Replicate measurements of absorption bands showed a standard error of $\pm$ 0.5%.

Quantitative infrared analysis of the lipid fractions was based on the measurement of three absorption bands, namely, the amide I band at 6.04 μ (1655 cm^{-1}), which all the sphingolipids have in common; the band at 10.3 μ (970 cm^{-1}), which is present in compounds containing a *trans* double bond; and finally the ester carbonyl band at 5.74 μ (1740 cm^{-1}), which appears in glycerophosphatide-contaminated fractions. The calibration curves of the bands at 6.04 μ, 10.3 μ, or 5.74 μ, which were derived from cerebrosides or sphingomyelins, and lecithin (isolated by chromatography), agreed closely with Beer's law.

The usefulness of the amide I band for analytical purposes was enhanced greatly by the finding of the similarity of its intensity in the two main types of sphingolipids, e.g., glycosphingosides and phosphosphingosides. Samples of 11 cerebroside and sphingomyelin preparations, which were isolated from five different brains each, showed average extinction coefficients of 1.22 and 1.29 arbitrary units, respectively. This finding allowed use of a common extinction coefficient for determination of both types of compounds. The correctness of this infrared method was further vali-

dated by the agreement of the results with chemical analysis of most of the lipid fractions.

The absorbance at 10.3 μ (970 cm^{-1}) was determined in sphingolipid fractions, in which it could be related to the presence of *trans* double bonds of a component (glycosphingoside) or to the lack of *trans* double bonds of a component (dihydroglycosphingoside) without being affected by overlapping phosphate absorptions such as occur in sphingomyelin. Calibrations of the band with standard cerebroside samples isolated from different brains agreed closely with one another and with Beer's law and showed average extinction coefficients (E = base line absorbence [10.3] per milligram cerebroside found by chemical analysis) which varied only slightly, e.g., from 0.28 to 0.30 arbitrary units. Since the extinction coefficient of a dihydrocerebroside must be zero, the relative cerebroside or dihydrocerebroside content of a mixture of the two components can be calculated from the equations:

$$\% \text{ cerebrosides} = \frac{\text{E of the unknown mixture}}{\text{E of cerebroside standard}} \times 100,$$

and

$$\% \text{ dihydrocerebroside} = 100 - \% \text{ cerebroside}.$$

The absolute values of the two components can be calculated from the "total" cerebroside figures obtained by chemical analysis or infrared measurement of the amide I band.

Measurement of the ester carbonyl band at 5.74 μ (1740 cm^{-1}) was standardized on phosphatidyl ethanolamine or lecithin isolated from brain and used for determination of contaminating glycerophosphatides. The determination of the components of binary mixtures was carried out in the conventional way (12, 13).

Paper Chromatography. Paper chromatography of the sugar components of glycosphingolipids was carried out essentially as described previously (14, 15, 16). A 1-mg sample of the lipid was hydrolyzed in a sealed tube with 2 N H_2SO_4 at 100° for 3 hours. The hydrolyzate was filtered for removal of fatty acids, neutralized with solid $BaCO_3$, and then filtered again. The filtrate was freeze-dried and the lyophilized material thus obtained dissolved in a small amount of distilled water. An aliquot of the solution was applied to strips of Whatman No. 1 paper. The chromatograms were developed in a butanol:pyridine:water system, air-dried, and finally sprayed with aniline hydrogen oxalate. Markers of pure sugars, such as glucose and galactose, were examined with each chromatogram.

Paper chromatography of sphingomyelin and contaminating glycerophosphatides was carried out by the procedure of Marinetti and Stotz (17). Replicate chromatograms were tested with ninhydrin reagent, Rhodamine 6-G, and appropriate reagents for choline. Unstained areas of other replicate strips of known R_f values were extracted with lipid solvents. The extracts were mixed with solid KBr for preparation of disks, which were examined by infrared spectroscopy. Extracts from plain strips treated in the same way were used as blanks for calculation of the infrared spectra (10).

Chemical Analysis. Chemical analysis supplemented infrared analysis and paper chromatography in searching for as many constituent radicals of the complex lipids as possible. Total lipid phosphorus was determined by the method of Sperry (18), alkali-stable phosphorus by the method of Schmidt *et al.* (19), hexose by the method of Brand and Sperry (20), phosphatidyl ethanolamine and serine by the method of Axelrod *et al.* (21), sphingosine by the methods of McKibbin and Taylor (22) and Sakagami (23), aldehydes by the method of Feulgen and Bersin (24), and total fatty acids of hydrolyzates by the method of Schmidt-Nielson (25). Nitrogen determinations were carried out by heating samples with concentrated H_2SO_4 in sealed Vycor glass tubes and subsequent nesslerization.

RESULTS

Chromatography of Brain Sphingolipids. The principal results of one fractionation with combined chemical, infrared, and weight determinations of the lipid fractions are summarized in Figure 1. The chemical data included in the illustration have been labeled to indicate the actual composition of the fractions only if it has been proved by a sufficient number of constituent radicals. General review of the chromatograms shows great constancy of the elution pattern, which allowed equating of solvent mixtures or fraction numbers used in different experiments. Comparison of the values of the weights, infrared and chemical determinations indicates the extent of agreement of the analytical data, which may be used as a suggestion of the correctness of the classification of the sphingolipid fractions. The chromatographic fractions presented in the illustrations can be divided into three groups of well-defined regularly occurring fractions of different sphingolipid composition, and several additional groups of less well-defined or less regularly occurring fractions.

Separation of Cerebrosides. The first group of fractions (Nos. 1 to 9) which was eluted with mixtures of chloroform-methanol ranging from 95:5 to 85:15

contained glycosphingosides. Some of the fractions showed a minimal phosphatide content. Greater amounts of contaminating phosphatides occurred in other fractionations in which a preceding hydrolysis step, as already described, was omitted. It is felt, however, that both procedures may be used for tentative characterization of the complex sphingolipid fractions.

Examination of the major fractions of the group (Nos. 6 to 8) shows agreement of the infrared and weight values with the chemical cerebroside figures derived from hexose determinations, thus suggesting that these major fractions were composed of rather pure glycosphingosides of the cerebroside class. The correctness of this assumption was proved further by chemical analysis of some of the fractions. Thus, fraction No. 7 of the experiment illustrated in Figure 1,

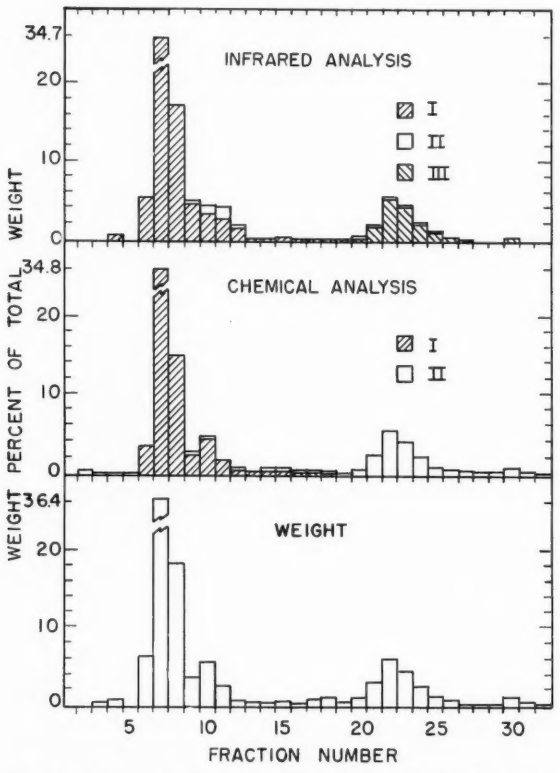

FIG. 1. Chromatographic separation of 325 mg of sphingolipids from white matter of post-mortem brain. Weight, infrared analyses (*I*, glycosphingosides; *II*, glycerophosphatides; and *III*, sphingomyelins), and chemical analysis; (*I*, cerebrosides; and *II*, phosphatides) are expressed as weight per cent of the sum of weights of all the fractions. The infrared measurements were converted into weights by use of the extinction coefficients of pure cerebrosides, sphingomyelins, and lecithins described in the text. The chemical data were converted into weights by multiplication of the analytical results by appropriate factors.

which weighed 160 mg, showed a cerebroside value of 150 mg when calculated from hexose determinations, and values of 156 mg or 160 mg when derived from sphingosine-nitrogen (2.6 mg) and fatty acid analysis (72.5 mg), respectively. These results confirm findings of Weiss (3) and preliminary reports of Schwarz *et al.* (1), and show that cerebrosides can be obtained in about as good a yield and purity by chromatography on silicic acid as by the methods of Radin *et al.* (4, 26) using Florisil and mixed ion exchange resins.

Further classification of the cerebroside fractions as phrenosine or kerasine must be deferred until gas-liquid chromatography of the fatty acids, now in progress, is completed. Separation of the normal (not hydroxylated) and hydroxy cerebroside acids carried out by the method of Kishimoto and Radin (4) showed that the first of the major cerebroside fractions (No. 7 of another fractionation) contained 76% normal and 24% hydroxy acids, while the following fraction (No. 8) was composed of 91% hydroxy and only 9% normal acids. Gas-liquid chromatography of the acid derivatives was then carried out with a Barber-Colman instrument. The normal and hydroxylated acids comprised a number of individual acids of the following chain length or retention times in minutes (t_R). The normal cerebroside acids of fraction No. 7 contained 44% acids of t_R 67 (C_{24}, lignoceric acid), 16% of t_R 48 to 17 (C_{20} and below), and 23% of t_R 9 and below, mostly stearic acid. The hydroxy acids of fraction No. 8 comprised 74% acids of t_R 86 (hydroxy lignoceric acid), two other acids amounting to 8% and 13% with t_R of 51 and 61, respectively, and two minor components with smaller t_R (even lower chain length). Further identification of these fatty acids will be carried out when additional pure standards are available. The results obtained so far agree with preliminary findings of Weiss (3) that a kerasine-containing normal fatty acid might be eluted from silicic-acid columns in front of phrenosine which contains hydroxylated acid, but stress that these two fractions are made up of a number of different fatty acids.

Galactose- and Glucose-Containing Sphingolipids. Paper chromatograms of the sugars which were isolated from the sphingolipid fractions (Nos. 4 to 18) of six different brains have been summarized in Figure 2. It may be noted from the figure that in addition to galactose, glucose, estimated as 24% of the total hexose, occurred in the sphingolipids of mixed gray and white matter of a brain of a 93-year-old. Definite but smaller amounts of glucose were found also in lipids of the white matter from a case of multiple sclerosis, while lipids of the white matter of "normal" brains contained only galactose.

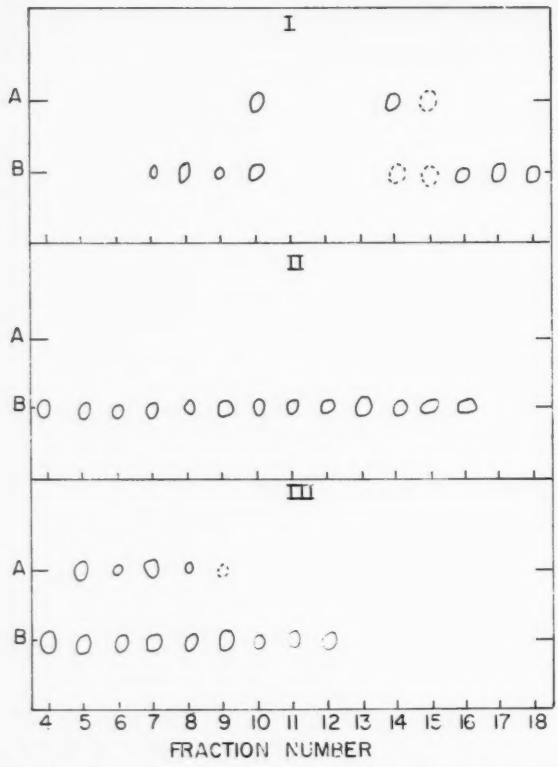

FIG. 2. Paper chromatograms of sugar preparations from the chromatographic brain sphingolipid fractions of (*I*) a case of multiple sclerosis, (*II*) five different patients who died free of nervous diseases, and (*III*) a 93-year-old. (*A*) R_f marker of glucose; (*B*) R_f marker of galactose.

Dihydrocerebrosides. The second group of fractions (Nos. 10 to 20), which was eluted with mixtures of chloroform-methanol ranging from 85:15 to 1:1 (v/v), contained glycosphingosides and small amounts of contaminating glycerophosphatides. The infrared spectra of the fractions were quite similar to those of cerebrosides, but the *trans* double bond absorption at 10.3 μ was often either absent or considerably reduced, suggesting the possible occurrence of dihydroglycosphingosides.

For further proof of this suggestion, the sphingolipid bases of this second group of fractions and of major cerebroside fractions (Nos. 7 and 8) were prepared, oxidized to aldehydes (23), and these aldehydes then examined by gas-liquid chromatography (27). The gas-liquid chromatograms showed that the aldehydes derived from the cerebroside fractions produced a major peak, probably related to the oxidation product of the sphingosine component, and a much smaller peak at about half of its retention time, probably related to small amounts of dihydrosphingosine. The chromatograms of the aldehydes from the second group of fractions, however, showed a reversed pattern, e.g., a minor peak at the longer retention time corresponding to the sphingosine and major peak at about half of the retention time, probably corresponding to the dihydrosphingosine content of the fractions. These results confirmed the suggestions from infrared spectroscopy that these fractions (Nos. 10 to 20) contained predominantly dihydroglycosphingosides.

Isolation of Sphingomyelin. The third group of fractions (Nos. 21 to 30), which was eluted with mixtures of chloroform-methanol ranging from 1:1 to 5:95 (v/v), and pure methanol, was composed mainly of rather pure phosphosphingosides. Preparations which included mild alkaline hydrolysis prior to the chromatography on silicic-acid columns yielded phosphosphingoside fractions of great purity, while isolations which omitted the hydrolysis step produced sphingosides which were often contaminated, particularly with lecithin.

The phosphosphingoside was identified as sphingomyelin by infrared spectroscopy, paper chromatography on silicic acid-impregnated paper, and chemical analysis. The infrared spectrum of such a fraction (No. 21, Fig. 1) showed the characteristic bands of pure sphingomyelin (1, 10). The paper chromatograms of the fraction exhibited a single choline-positive spot at the R_f value of pure sphingomyelin. The chemical analysis of a somewhat larger fraction (No. 22, Fig. 1) showed almost negative ninhydrin reaction. The phosphorus content of 3.79%, nitrogen value of 3.2%, and sphingosine content of 30.33% found in this fraction furthermore agree with its designation as sphingomyelin. These results show that chromatography on silicic-acid columns may be used for preparation of pure sphingomyelins from relatively small amounts of brain tissue.

Other Sphingosine Derivatives. Previous studies (28, 29) showed that relatively small molecular-weight derivatives of sphingosine could be prepared by partial hydrolysis of cerebrosides and sphingomyelin, and that the ceramide, N-lignoceryl sphingosine, might be isolated even from unhydrolyzed lipids of spleen (30), liver (31), and red cells (32). There is, however, no definite evidence indicating that such sphingosine derivatives occur in brain tissue. In the present study, 21 brain sphingolipid fractionations were carried out. There were some analytical discrepancies, probably explainable in part by contaminations or analytical errors. Only two fractionations of brain lipids from cases of multiple sclerosis suggested the occurrence of ceramide-like substances.

In the first case, the fractionation of a large part of

the brain yielded a fraction No. 5, weighing 8.8 mg. It contained no hexose, not more than a trace of phosphorus (0.16%), 1.6% nitrogen, and a molar sphingosine-fatty acid ratio of 1:0.95, strongly suggesting the occurrence of a ceramide. The correctness of this suggestion was further stressed by the elution of 98% of a synthetic N-palmityl sphingosine (prepared by Dr. H. Carter and obtained from Dr. P. Kennedy) in similar chromatographic fractions (Nos. 4 and 5), and by the great similarity of the infrared spectra of both the synthetic and the brain ceramide fraction (Fig. 3). The second brain with multiple sclerosis showed a fraction No. 9 with sphingosine and fatty acid values roughly adequate for a cerebroside, but less than half of the hexose required, suggesting the occurrence of a cerebroside-ceramide mixture.

Additional sphingosine derivatives resembling mucolipids were eluted in front of the main sphingomyelin fractions (Nos. 19 to 24), particularly when lipid preparations of large amounts of gray matter were chromatographed without subjecting them to the washing procedure (8) prior to the separation. Other unidentified substances showing less phosphorus and less choline occurred at the tail of the phosphosphingoside fractions of multiple sclerosis brains. These fractions require further examination.

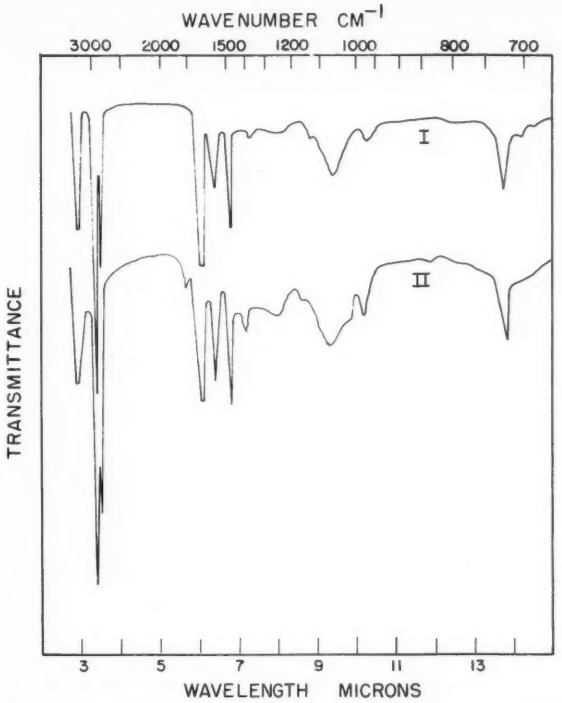

FIG. 3. Infrared spectra of (*I*) N-palmityl sphingosine and (*II*) an aliquot of the chromatographic sphingolipid fraction No. 5 from a case of multiple sclerosis (KBr disks).

DISCUSSION

The results obtained in this investigation give conclusive evidence that chromatography on silicic-acid columns, particularly when preceded by mild alkaline hydrolysis, is an effective means of isolation of glycosphingosides and phosphosphingosides. Good recoveries of hexose and alkali-stable, phosphorus-containing lipids from the hydrolyzates showed that the hydrolysis, while effectively destroying the contaminating phosphatides, did not too greatly alter the molecular structure of the sphingolipids. The principal results, furthermore, were checked on sufficiently pure nonhydrolyzed materials.

Pure glycosphingosides of the galactocerebroside class were isolated, and in part further characterized by analysis of their fatty acid components. Studies of cerebroside fatty acids of diseased human brain are in progress. Significant amounts of glucocerebrosides detected in the brain of an old patient, and smaller amounts of it found in brain with multiple sclerosis, show the importance of further examination of the sugar component of the cerebrosides. The described identification of a small ceramide fraction comprising about 1.4% of the total sphingolipids of a brain with multiple sclerosis should stimulate further search for ceramides in diseased brain tissue. The finding of a dihydroglycosphingoside in human brain, previous discovery of dihydrosphingosine in beef brain (27, 33, 34), and very recent detection of a C_{20}-sphingosine in beef and horse brain (35) stress the importance of analysis of the bases of the complex sphingolipids of the brain. Examination of the probable occurrence of sulfatides and other not yet characterized lipid components of some chromatographic fractions is in progress. The simple methods of preparation and assay described in this paper will enhance more complete characterization of sphingolipids occurring in tissues.

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Wheat flour lipids: II. isolation and characterization of glycolipids of wheat flour and other plant sources*

H. E. Carter, K. Ohno,† S. Nojima,‡
C. L. Tipton,§ and N. Z. Stanacev‖

Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana, Illinois

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SUMMARY

Methods involving solvent fractionation and silicic-acid column chromatography have been developed for isolating monogalactosyl- and digalactosylglycerol lipids and cerebrosides from the benzene-extracted lipids of wheat flour. A comparative study has been made of the composition of the lipid mixtures obtained from bleached and unbleached flours. The lipids from bleached flour were found to contain covalently bound chlorine, apparently resulting from reaction with chlorine, the bleaching agent. A preliminary study of the occurrence of galactosylglycerol lipids in other plant materials is reported.

In a previous paper (1) we reported the preparation from wheat flour lipids of a crude mixture of glycolipids which, on alkaline hydrolysis, yielded among other substances, monogalactosyl- and digalactosylglycerol. The structures of the two glycerol derivatives were established as β-D-galactopyranosyl-1-glycerol and α-D-galactopyranosyl-(1 → 6)-β-D-galactopyranosyl-1-glycerol. A partial separation of the intact galactosylglycerol lipids was achieved by virtue of the greater solubility in organic solvent of the monogalactosylglycerol lipid as compared to the digalactosylglycerol lipid.

During the past three years an extensive study has been made of the composition of wheat flour lipids. A simple procedure has been devised for preparing a glycolipid fraction relatively free of triglyceride and sterol ester, and chromatographic procedures have been developed for separation of the glycolipids into relatively pure monogalactosyl- and digalactosylglycerol lipid fractions and a crude cerebroside fraction.

These studies are described in the present paper, together with data on the comparative composition of lipids from bleached and unbleached wheat flour.

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† Postdoctoral Research Associate in Chemistry. Present address: Sapporo Medical College, Sapporo, Japan.

‡ Rockefeller Foundation Research Fellow, 1958–59, Postdoctoral Research Associate, 1959–60. Present address: Department of Biochemistry, National Institute of Health, Tokyo, Japan.

§ Research Assistant in Chemistry.

‖ Postdoctoral Research Associate in Chemistry.

EXPERIMENTAL AND RESULTS

Analytical Methods. Elementary analysis for C, H, Cl, and N (Dumas) was obtained. Phosphorus was determined by the method of Harris and Popat (2), total nitrogen by the micro-Kjeldhal method, and the long-chain base nitrogen by the method of McKibbin and Taylor (3). Sugars were determined by the method of Radin *et al.* (4).

The infrared spectra were obtained from smeared material on a Perkin-Elmer 21 double beam spectrophotometer, using sodium chloride prism.

The conditions for gas chromatographic analysis are described in the experimental part.

Unbleached Wheat Flour. Isolation of Monogalactosylglycerol Lipid, Digalactosylglycerol Lipid, and Cerebrosides. One hundred pounds of unbleached wheat

flour[1] was extracted with benzene at room temperature, courtesy of the Procter and Gamble Company. The benzene extract was concentrated *in vacuo*, and the residual heavy syrup was stored in the cold room at 4°.

In a typical experiment, 1 liter of benzene extract (containing about 280 g of lipid), was treated with 10 liters of acetone. After standing overnight at 4°, the precipitate was filtered, redissolved in 100 ml of benzene, and reprecipitated with 1 liter of acetone. The insoluble material (BAI)[2] was filtered (12.3 g), and the filtrate was combined with the previous one.

The combined benzene-acetone solutions, after evaporation of solvent *in vacuo* at room temperature, gave 241.6 g of benzene-acetone soluble (BAS) yellow oil. This material was dissolved in 10 volumes of *n*-heptane (pre-equilibrated with 95% methanol) and extracted twice with equal volume of 95% methanol (pre-equilibrated with *n*-heptane). The methanol-soluble material (BAS-MS) was obtained by evaporation of the solvent, giving 30.8 g of brown oil. The heptane-soluble material (BAS-HS) was obtained in a similar manner, yielding 192.5 g of brown oil.

The analyses summarized in Table 1 show a clean separation of carbohydrate-containing material in the methanol phase. The heptane phase contained, mainly, triglyceride plus sterol and sterol ester.

For silicic-acid chromatography, 30.7 g of BAS-MS was dissolved in 100 ml of chloroform and applied to a 1-kg silicic-acid column (35 × 7 cm). Silicic acid (Mallinckrodt, 20% maximum water content, 100 mesh) was activated by heating in an oven for 12 hours at 110°. This activated silicic acid was washed thoroughly with methanol until the supernatant became clear. The slurry was then poured into a glass column and washed with 3 volumes of chloroform, after which the sample solution was applied. The elution was started with chloroform and continued with increasing concentrations of methanol in chloroform (0% to 30%); 500-ml fractions were collected. Table 2 contains data on the resultant combined fractions. The characterization of each fraction was carried out by paper chromatography. After mild alkaline hydrolysis (1 N sodium hydroxide at 37° for 24 hours), followed by neutralization with Dowex-50 (HCO_3^-), the water-soluble material was applied to Whatman No. 1 filter paper and chromatographed in an *n*-butanol:pyridine:water (6/4/3, v/v) solvent system, using the ascending technique. Standards of glycerol, monogalactosylglycerol, and digalactosylglycerol were applied under the same conditions. The combined fractions, listed in Table 2, were analyzed in a similar manner and also by infrared spectroscopy.

[1] General Mills Soft-as-Silk unbleached cake (wheat) flour.

[2] Abbreviations used: BAI: benzene-acetone insoluble material; BAS: benzene-acetone soluble material; BAS-MS: benzene-acetone soluble-methanol soluble material; BAS-HS: benzene-acetone soluble-heptane soluble material.

TABLE 1. ANALYSES OF HEPTANE- AND METHANOL-SOLUBLE MATERIAL FROM BENZENE-ACETONE SOLUBLE EXTRACT OF UNBLEACHED WHEAT FLOUR

	Heptane Soluble (BAS-HS)	Methanol Soluble (BAS-MS)
	per cent	*per cent*
Sugar (anthrone)	negative	13.3
Nitrogen (Kjeldhal)	0.03	0.11
Long-chain base nitrogen (McKibbin-Taylor)	0.02	0.17
Phosphorus	0.01	0.12

On the basis of these analyses, fractions 1 to 29 contained neutral fats; fractions 34 to 40 were rich in the monogalactosylglycerol lipid; digalactosylglycerol lipid was concentrated in fractions 44 to 72, and cerebrosides in fractions 61 to 65. The recovery was 82.3% of the starting material.

Purification of Monogalactosylglycerol Lipid. A pure sample of monogalactosylglycerol lipid was obtained by rechromatography of combined fractions 34 to 40. This material (5.69 g) was dissolved in a minimum amount of chloroform and applied to a 200-g column of silicic acid (3.5 × 50 cm). Two hundred and ninety fractions (each 12.5 ml) were eluted using chloroform (fractions 1 to 140) and chloroform-methanol mixtures (fractions 141 to 290), as solvents. Monogalactosylglycerol lipid, free from the neutral glycerides and

TABLE 2. SILICIC-ACID CHROMATOGRAPHY OF METHANOL-SOLUBLE MATERIAL (30.7 G) FROM BENZENE-ACETONE SOLUBLE EXTRACT OF UNBLEACHED WHEAT FLOUR

Combined Fractions	Solvent (Chloroform:-Methanol)	Weight	Paper Chromatographic Analysis*		
			Glycerol	Mono-galactosylglycerol	Di-galactosylglycerol
		g			
1–29	100:0	10.76	+++	+	−
30–33	98:2	0.11	++	+	−
34–40	98:2	5.69	+−	+++	−
41–43	98:2	0.56	+−	+++	−
44–60	96:4	0.91	+−	+	+++
61–65	94:6	0.60	+−	−	++
66–72	90:10	5.11	−	−	+++
73–80	80:20	1.30	−	−	+−
81–91	70:30	0.24	−	−	−

* Abbreviations: +++ strongly positive; ++ positive; + slightly positive; − negative. Detected with permanganate-periodate spray.

digalactosylglycerol lipid, was accumulated in fractions 181 to 289 (eluted with chloroform:methanol 98/2, v/v). These combined fractions, after evaporation of solvent, gave 1.88 g of white amorphous powder containing 23.9% of sugar. The infrared spectrum showed strong absorption due to hydroxyl and ester bonds. Paper chromatographic analysis of the alkaline hydrolysis products, as described above, showed only the presence of monogalactosylglycerol (detected with permanganate-periodate spray).

Purification of Digalactosylglycerol Lipid. The combined fractions 66 to 72 (Table 2) showed only digalactosylglycerol in paper chromatography, but in the infrared spectrum, absorptions due to free fatty acids (1705 cm^{-1}) and cerebrosides (1525, 1650 cm^{-1}) were also present. A solution of 5.10 g of the combined fractions 66 to 72 in 50 ml of 95% methanol was passed three times through an Amberlite®-MB-3 column (30 × 4.5 cm). The column was washed with 500 ml of the same solvent. The combined methanolic solutions were evaporated to dryness *in vacuo* at room temperature, giving 3.34 g of white amorphous, rather hygroscopic material. The analysis showed 33.5% of carbohydrate, and in the infrared spectrum the absorption due to free acids was completely absent. However, the absorption due to amide bonds (1525, 1650 cm^{-1}) was still present, indicating (on the basis of ratio between ester and amide bonds) 5% to 10% of cerebrosides as impurity. The *complete* separation of digalactosylglycerol lipid from cerebrosides was achieved only on partially methylated material, as is described in the next article in this periodical (5).

Saponification Equivalents of Mono- and Digalactosylglycerol Lipids. Saponification equivalents were determined on 100 to 150 mg samples of purified monogalactosylglycerol lipid, and on 200 to 400 mg samples of digalactosylglycerol lipid. A solution of 139.8 mg of monogalactosylglycerol lipid in 10 ml of 0.1 N methanolic sodium hydroxide and 1 ml of water was refluxed for 2 hours. The excess of sodium hydroxide was determined by titration with 0.1 N hydrochloric acid, using a pH meter for estimation of the end point. The consumption was 3.70 ml 0.1 N sodium hydroxide. For digalactosylglycerol lipid, a solution of 378.8 mg of sample in 10 ml of 0.1 N methanolic sodium hydroxide and 1 ml of water was refluxed for 2 hours, consuming 8.14 ml of base. The saponification equivalent for monogalactosylglycerol lipid obtained in this way was 378, and for digalactosylglycerol lipid 466, giving molecular weights, respectively, of 378 and 466 (one fatty acid per molecule) or 756 and 932 (two fatty acids per molecule). (Calculated saponification equivalents for monostearoyl-monogalactosylglycerol, 520.7; distearoyl-monogalactosylglycerol, 393.5; monostearoyl-digalactosylglycerol, 682.8; distearoyl-digalactosylglycerol, 474.6.)

For determination of neutralization equivalents, fatty acids were extracted from the acidified aqueous-methanolic saponification mixture with ether. The ether solution was washed with water, dried, and evaporated; the resulting mixture of fatty acids was dried *in vacuo* and titrated with 0.1 N sodium hydroxide. For monogalactosylglycerol lipid a value of 258, and for digalactosylglycerol lipid, a value of 303 was obtained.

Composition of Fatty Acids of Mono- and Digalactosylglycerol Lipid. The fatty acids, obtained as described above, were converted to methyl esters with diazomethane. The gas-liquid chromatographic analyses of methyl esters were performed in the Research and Development Department of the Procter and Gamble Company, Cincinnati, Ohio. Conditions for the analysis: The adipic acid-ethylene glycol polyester (12% on 80 to 100 mesh Chromosorb W) column (200 cm); helium flow rate, 91 ml/minute; column temperature, 208°; sample volume, 0.4 ml of 50% solution in methanol. The results of the analyses are given in Table 3.

TABLE 3. ANALYSES OF METHYL ESTERS OF FATTY ACIDS FROM GALACTOSYLGLYCEROL LIPIDS FROM UNBLEACHED WHEAT FLOUR

Corresponding Acid	Corrected Retention Time	Composition*	
		Mono-galactosylglycerol lipid	Di-galactosylglycerol lipid
	minutes	*per cent*	*per cent*
Myristic	4.39	0.5	
Palmitic	8.76	13.9	41.6
Palmitoleic	9.55	3.4	
Stearic	16.94	1.3	4.4
Oleic	18.76	17.2	12.1
Linoleic	22.73	57.0	29.3
Linolenic	28.49	2.0	
All others		5.1	12.6

* Values listed can be in error as much as ±15% relative.

Isolation of Crude Cerebroside Fraction. Combined fractions 61 to 65 (600 mg), obtained by silicic-acid chromatography of benzene-methanol soluble material, as described above, were dissolved in 50 ml of 1 N sodium hydroxide in aqueous methanol and left at 37° for 36 hours. After dilution with the same volume of water and neutralization with 1 N hydrochloric acid to pH 7 (in ice), the suspension was extracted three times with chloroform. The extract was washed with water, dried, and evaporated to dryness. The residue was redissolved in about 100 ml of 95% methanol and the solution was passed through a Dowex-2 (OH^-) column to remove fatty acids. After evaporation of

solvent, 297 mg of colorless powder (m.p. 159°–162°) was obtained (analyses: C 66.72, H 11.09, N 2.00%).

The infrared spectrum showed strong absorption due to hydroxyl, amide-, and *trans*-double bonds, and complete absence of ester bonds.

Isolation of Sitosteryl-β-D-Glucoside. Fractions eluted with chloroform:methanol 98/2 (v/v) from silicic acid in the course of chromatography of methanol-soluble material from benzene extracts of unbleached wheat flour were contaminated with Liebermann-Burchard positive material. This material was separated in the course of mild alkaline hydrolysis of fractions, described above, as methanol and acetone-insoluble material. For purification, in one experiment, 500 mg of this material was suspended in chloroform and applied to a silicic-acid column (16 × 2.5 cm). In fraction 3, eluted with chloroform:methanol 97/3 (v/v), 159.8 mg of material was recovered. The elementary analysis of this material indicated the presence of sitosteryl-β-D-glucoside (6).

$C_{35}H_{60}O_6$ (576.83)

Calculated:	C 72.88, H 10.50%
Found:	C 72.02, H 10.39%

Acidic hydrolysis of material released glucose, as detected by paper chromatography.

The polyacetyl derivative of the same material, prepared in the usual way, after crystallization from *n*-hexane, gave a crystalline compound (m.p. 166°–168°). The reported melting point for tetraacetyl-sitosteryl-β-D-glucoside is 166°–168°(7). The elementary analyses were in fair agreement with calculated values:

$C_{43}H_{68}O_{10}$ (744.97)

Calculated:	C 69.32, H 9.20%
Found:	C 68.78, H 9.12%

The infrared spectrum of this compound was superimposable with that of authentic sample.

Degradation of Sitosteryl-Glucoside to Sitosterol. To a solution of 52 mg of sitosteryl-glucoside, isolated as described above, in 4 ml of chloroform and 4 ml of methanol, 120 mg of periodic acid in 5 ml of water was added. After standing overnight at room temperature, the reaction mixture was passed through Dowex-2-(OH^-) column. To the clear filtrate a mixture of 50 mg of hydroxylamine hydrochloride and 50 mg of sodium acetate was added, and the solution was left overnight at room temperature. After deionization and evaporation to dryness, 52 mg of crude oxime was obtained, which was dissolved in 0.3 N hydrochloric acid in methanol-chloroform 1/1 (v/v) and left at 30° for 24 hours. The reaction mixture was extracted with ether. The ether solution was evaporated to dryness and the residue (36 mg), after two recrystallizations from ethanol, gave 20 mg of crystalline compound melting at 142° (β-sitosterol, m.p. 137°–137.5° [8]).[3] The elementary analyses were in good agreement with the calculated values for sitosterol with 1 mole of water:

$C_{29}H_{52}O_2$ (432.91)

Calculated:	C 80.31, H 12.08%
Found:	C 81.01, H 11.70%

Bleached Wheat Flour.[4] *Isolation and Characterization of Lipids.* The benzene extract of bleached wheat flour, obtained in the same way as described for unbleached wheat flour, was precipitated with acetone. To the solution of about 600 g of lipid in 900 ml of benzene, 9 liters of acetone was added, and after standing at 4° overnight, the precipitate was filtered, redissolved, and reprecipitated in the same way. The insoluble material (130 g) was filtered off, and the benzene-acetone soluble material was obtained by evaporation of solvent. This brown oil (430 g) was distributed between 95% methanol and *n*-heptane, as described above, giving, after evaporation of solvents, 192 g of BAS-MS and 213 g of BAS-HS. The comparative yields of these fractionations are listed in Table 4.

TABLE 4. COMPARATIVE YIELDS OF VARIOUS FRACTIONS AFTER SOLVENT FRACTIONATION OF BENZENE EXTRACT OF UNBLEACHED AND BLEACHED WHEAT FLOUR LIPIDS

Material	BAS	BAS-HS	BAS-MS	BAI
	per cent	*per cent*	*per cent*	*per cent*
Unbleached wheat flour	73–93 (78)*	43–81 (43)*	12–30 (15)*	21–23
Bleached wheat flour	71–86 (86)*	42–58 (50)*	27–35 (34)*	14–23 (17)*

* Figures in parentheses are values most frequently obtained.

The silicic-acid chromatography of 50.0 g of BAS-MS material on 1 kg of silicic acid (35 × 7 cm), under the previously described conditions, gave fractions which were characterized as listed in Table 5. On the basis of these analyses (combined with results obtained by infrared spectroscopy), fractions 1 to 5 were shown to be rich in neutral fats, fractions 13 to 24 in monogalactosylglycerol lipid, and fractions 33 to 71 in digalactosylglycerol lipid. The main cerebroside fraction appeared in fractions 66 to 71, although absorptions (at 1650 to 1660 and 1530 to 1550 cm^{-1}) due to amide bonds in the infrared spectrum were weaker and less

[3] The observed higher melting point probably indicates the presence of α-sitosterol as an impurity (8).

[4] General Mills Soft-as-Silk cake flour milled from soft red winter wheat and bleached with chlorine.

TABLE 5. SILICIC-ACID CHROMATOGRAPHY OF METHANOL-SOLUBLE MATERIAL (50.0 G) FROM BENZENE-ACETONE SOLUBLE EXTRACT OF BLEACHED WHEAT FLOUR

Combined Fractions	Solvent (Chloroform:-Methanol)	Weight	Paper Chromatographic Analysis*		
			Glycerol	Monogalactosylglycerol	Digalactosylglycerol
1–5	100:0	19.83	+++	−	−
11–12	99:1	1.67	+	+++	−
13–18	98:2	4.34	+	+++	−
19–24	97:3	2.73	+	++	−
25–30	96:4	2.50	++	++	++
31–36	95:5	4.59	−	+	++
37–42	94:6	2.64	−	+−	+++
43–48	93:7	0.79	−	+−	+++
49–54	92:8	0.59	−	+−	+++
55–60	90:10	1.15	++	−	+++
61–65	80:20	0.97	+	−	+++
66–68	80:20	1.09	+	−	+++
69–71	50:50	0.64	−	−	+++

* Abbreviations: +++ very positive; ++ positive; + slightly positive; − negative. Detected with permanganate-periodate spray.

sharp than in the case of the cerebroside fraction isolated from unbleached wheat flour. Recovery was 89% of starting material.

Comparative analytical data of the various fractions from silicic-acid chromatography of bleached and unbleached material are listed in Table 6.

Benzene-Acetone Insoluble Fractions of Unbleached and Bleached Wheat Flour Lipids. Benzene-acetone insoluble materials (BAI) of unbleached and bleached wheat flour lipids were distributed between 95% methanol and *n*-heptane by dissolving the material (16.83 g of material from unbleached, and 67.0 g of material from bleached wheat flour) in 95% methanol, and extracting the solution several times with *n*-heptane. Results of these distributions are given in Table 7, showing a low yield of heptane-soluble material from unbleached wheat flour in comparison with the high yield from bleached wheat flour. The methanol-soluble material of unbleached wheat flour is far less rich in sugar than the corresponding fraction from bleached flour.

The formation of interfacial material, not soluble in methanol or heptane, was observed. The amount of interfacial material from unbleached wheat flour lipid was 53%, and from bleached wheat flour lipid 49.3% (variation in range of 17% to 50%). This material, with content of phosphorus in range of 0.60% to 1.00% and nitrogen 7% to 11%, is lipoprotein or lipopeptide-like material, a detailed study of which fraction will be the subject of a subsequent communication.

TABLE 6. COMPARATIVE ANALYTICAL DATA ON FRACTIONS ELUTED FROM SILICIC-ACID CHROMATOGRAPHY OF METHANOL-SOLUBLE MATERIAL FROM BENZENE-ACETONE SOLUBLE EXTRACT OF UNBLEACHED AND BLEACHED WHEAT FLOUR LIPIDS

Fraction (Chloroform:-Methanol)	Analyses						
	Sugar		Nitrogen		Phosphorus		Chlorine
	Unbleached	Bleached	Unbleached	Bleached	Unbleached	Bleached	Bleached Only
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
100:0	1.23	0.31	0.07	0.01	0.07	0.02	7.75
99:1	20.00	11.91	0.07	0.21	0.30	0.08	*
98:2	19.33	11.63	0.17	0.30	0.16	0.38	5.15
96:4	32.93	15.90	0.36	0.28	0.13	0.48	*
95:5	35.36	26.39	0.10	0.33	0.06	0.18	4.01
90:10	*	37.65	*	0.20	*	0.12	*
80:20	*	37.21	*	0.60	*	0.29	5.74
60:40	4.37	26.04	1.43	2.00	2.09	1.96	*

* Not determined on this fraction.

TABLE 7. SOLVENT DISTRIBUTION OF BENZENE-ACETONE INSOLUBLE MATERIAL OF UNBLEACHED (16.83 G) AND BLEACHED (67.0 G) WHEAT FLOUR LIPIDS*

Phase	Transfer Number	Yield		N		P		Sugar		Liebermann-Burchard Test	
		U	B	U	B	U	B	U	B	U	B
		g	*g*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
Heptane	1	0.09	8.75							+++	+++
Heptane	2	0.04	0.33							++	+++
Heptane	3	0.03	0.13							+	+++
Heptane	4	0.02	0.08							+	++
Methanol	5	0.09	0.19	1.77	1.80	1.96	1.11	3.21	9.24	−	−
Methanol	6	0.76	0.51	2.94	2.63	2.52	1.21	4.80	13.60	−	−
Methanol	7	6.37	20.04	3.71	4.48	2.25	1.17	†	15.82	−	−
Methanol	8	9.01	11.13	7.34	4.17	1.62	1.03	†	8.70	−	++
Methanol	9		3.41		5.17		1.20		8.65	−	+
Methanol	10		18.49		7.50		0.76		19.88	−	−

* Abbreviations: U = unbleached; B = bleached; +++ very positive; ++ positive; + slightly positive; − negative.
† Not determined on this fraction.

Isolation of Cerebroside-like Compounds from Unbleached Material. Methanol-soluble material from BAI of unbleached wheat flour (combined fractions 6 and 7, Table 7) was fractionated on silicic acid. The solution of 6.32 g of this material in chloroform: methanol 9/1 (v/v) was applied to a 200-g silicic-acid column. The fractions obtained (77% recovery), using solvent mixtures of chloroform-methanol (9/1 to 0/10, v/v), were analyzed after acidic hydrolysis, giving the sugars listed in Table 8. The infrared spectrum showed still very strong ester absorption. In order to obtain purified cerebrosides, fractions 1 and 2 from Table 8 were combined and hydrolyzed in 0.1 N aqueous sodium hydroxide at 37° for 33 hours. After cooling, the alkaline solution was neutralized with Amberlite® IR-120 (H^+) and repeatedly extracted with chloroform and methanol, giving 992 mg of waxy material. After trituration with acetone, 840 mg of acetone-insoluble substance was obtained, which still showed an ester absorption band in the infrared (1725 cm^{-1}). This material was again hydrolyzed at 37° for 14 hours, but in 1 N potassium hydroxide in methanolic solution. The solution was treated as above, giving 387 mg of ester-free material. This crude fraction was a mixture of manno-, gluco-, and possibly galactocerebrosides, but preliminary attempts to separate these cerebrosides were unsuccessful. Further studies on this fraction are under way.

TABLE 8. SILICIC-ACID CHROMATOGRAPHY OF METHANOL-SOLUBLE MATERIAL (6.32 G) FROM BENZENE-ACETONE INSOLUBLE EXTRACT OF UNBLEACHED WHEAT FLOUR LIPIDS

Fraction	Solvent	Weight	Analysis			Paper Chromatographic Analysis		
	Chloroform:-Methanol		P	N	Sugar	Mannose	Glucose	Galactose
		g	*per cent*	*per cent*	*per cent*			
1	9:1	1.931	2.21	0.91	7.28	−	+	+
2	8:2	0.594	2.23	1.62	14.92	+	−	+
3	7:3	0.555	3.04	2.80	6.51	−	−	+
4	6:4	0.387	2.87	4.03	*	−	−	+
5	1:1	0.493	2.63	3.67	22.20	−	+	+
6	0:10	0.587	2.87	4.28	*	−	−	+
7	0:10	0.220	2.59	4.68	*	Not determined		

* Atypical color with anthrone test

Isolation of the Cerebroside-like Compounds from Bleached Material. The methanol-soluble material from BAI of bleached wheat flour (combined fractions 6 and 7, Table 7) was chromatographed over silicic acid. Twenty grams of this material was applied to a 600-g silicic-acid column. The fraction eluted with chloroform:methanol 7/3 (v/v) gave 9 g of material with 22.1% of sugar, 1.03% of phosphorus, 0.99% of nitrogen, and 0.17% of long-chain base nitrogen. From two similar preparations 13.6 g of such material was obtained with strong ester absorption in the infrared spectrum. These combined materials were hydrolyzed with mild alkali (aqueous methanolic 0.1 N potassium hydroxide, 37°, 22 hours), and the solution was repeatedly extracted with chloroform. After evaporation of solvent, 9.6 g of a crude mixture of cerebrosides was obtained from bleached wheat flour. By fractionation over a 400-g silicic-acid column the chloroform-methanol 9/1 (v/v) fraction contained 1.05 g of glucocerebrosides; the chloroform-methanol 8/2 (v/v) fraction gave 1.26 g of mannocerebrosides. Both cerebrosides were characterized by infrared spectra and by paper chromatography of water-soluble acid hydrolysis products.

The crude mannocerebroside fraction (1.2 g) was triturated with acetone and centrifuged. The insoluble cerebrosides (580 mg) were dissolved in a minimum amount of cold methanol, and the insoluble material was filtered. The clear filtrate was diluted with acetone and cooled at 4°, giving 240 mg of white powder melting at 160°–200°. (Analyses: C 61.11, H 9.39, N 1.13, Cl 2.37, and sugar 45.77 and 47.11%.) These data are in reasonable agreement with a lignocerylsphingosine trimannoside structure (47.5% sugar; dimannoside, 37% sugar). This material, after acidic hydrolysis, gave a sharp and intensive spot for mannose, but glucose in very low concentration was also detected.

Distribution of chlorine in this material was determined by hydrolysis with 10% methanolic sulfuric acid for 4.5 hours in a sealed tube. After cooling and evaporation of methanol, the reaction mixture was extracted with *n*-hexane. The hexane solution was concentrated to dryness, yielding 27 mg of fatty acids, with 1.96% of chlorine.

The aqueous solution was made alkaline to pH 13 and repeatedly extracted with ether. The ether solution was washed with water, dried, and evaporated to dryness, giving 34 mg of long-chain base(s) with 2.99% of chlorine.

The remaining aqueous solution, after extraction with ether and isolation of long-chain base(s), was deionized with Amberlite®-IR-120 (H^+) and Dowex-2 (CO_3^-) resin. For complete hydrolysis of glucosidic linkage, this solution was again hydrolyzed with sulfuric acid (0.5 N, 12 hours) in a sealed tube, neutralized with Dowex-2 ($CO_3^=$), and evaporated to small volume. Four drops of phenylhydrazine were added and the reaction mixture was shaken vigorously for a few minutes and left standing at room temperature for a few hours. The crystalline material (4 mg) was

filtered and, after recrystallization from methanol, melted at 190°–191° (decomp.). The reported melting point for D-mannosephenylhydrazone is 195°–200° (decomp.) (9). The analysis was:

$C_{19}H_{18}N_2O_5$ (270.28)

Calculated: N 10.36%
Found: N 11.34%

Detection of Mono- and Digalactosylglycerol in Various Plants Material. Whatman No. 1 filter paper was used for ascending chromatography in *n*-butanol:pyridine:water 6/4/3 (v/v) solvent system. Permanganate-periodate spray was used for the detection of spots.

Corn phosphatides[5] (11.6 g) were extracted three times with dry acetone, yielding 6.13 g of acetone-soluble material and 5.19 g of acetone-insoluble fraction. Portions of these materials (1.85 g of acetone-soluble and 1.88 g of acetone-insoluble material) were refluxed with 30 ml of 0.1 N sodium hydroxide for several hours, then acidified to pH 1 with 1 N hydrochloric acid, and extracted several times with ether. The water solution was deionized by passing through Amberlite®-MB-3 column. After evaporation of the solvent, 62 mg of material from the acetone-soluble lipids and 75 mg of material from the acetone-insoluble lipids were obtained. These materials were redissolved in a small amount of water and chromatographed as described above. In the acetone-insoluble fraction, digalactosylglycerol was detected; in the acetone-soluble fraction, only monogalactosylglycerol was present.

Corn gluten[6] (75 g) was extracted with benzene for 48 hours in a Soxhlet apparatus. After concentration of benzene to dryness, 3.70 g of brown oil was obtained. A portion of this material (0.83 g) was hydrolyzed with alkali and treated as described above. The water-soluble material (21 mg), on paper chromatography, gave spots corresponding to the monogalactosylglycerol and digalactosylglycerol.

Wheat germ oil[7] (2.93 g) was hydrolyzed in the same manner as described above, giving 69 mg of water-soluble material. This material, on paper chromatography, gave digalactosylglycerol.

Euglena gracilis, strain Z, was grown on a sucrose-containing medium (10) in the light and the lipids were extracted with chloroform:methanol 2/1 (v/v). After evaporation of solvent, the crude lipids were treated with acetone. The acetone-insoluble lipids were hydrolyzed with alkali and chromatographically examined. In the water-soluble material, monogalactosylglycerol was detected.

After similar treatment of organism grown on the same medium in the dark, no galactosylglycerol could be detected.

Green oat groat oil[8] (1.01 g) was heated on a steam cone for 3 hours with 30 ml of 0.1 N sodium hydroxide, cooled, acidified with 6 N hydrochloric acid, and extracted with ether. The aqueous solution was then deionized by passing over an Amberlite®-MB-3 column and the effluent lyophilized, yielding 0.041 g of material. This material was only partially soluble in water. The paper chromatography revealed the presence of a large amount of digalactosylglycerol, a trace of monogalactosylglycerol, glycerol, and an unidentified material with lower R_f than digalactosylglycerol.

DISCUSSION

In the previous paper (1) preliminary evidence was presented that benzene-extracted wheat flour lipid contains glycerides, sterol and sterol derivatives, lipids containing galactosylglycerol, and a lipoprotein (peptide?) fraction. In undertaking a study of the glycolipid fraction, our first efforts were directed to the development of simple procedures for preparing a glycolipid fraction relatively free of other constituents.

The main part of the peptide-containing material could be removed from the benzene-extracted lipids by precipitation with acetone. The precipitate contained the majority of the amino acid nitrogen together with a considerable amount of lipid, some of which could be readily separated by repeated precipitation, giving a lipopeptide fraction with lipid more firmly bound.

Distribution of the benzene-acetone soluble material between *n*-heptane and methanol gave a sharp separation of glycolipids in the methanol phase, with triglycerides and sterol esters concentrated in the heptane phase (Table 1). The methanol-soluble material was rich in sugar-containing substances (Table 1), and was used as the starting material in most of the further fractionation studies. A number of samples of wheat flour lipid obtained from both bleached and unbleached hard and soft flour have been investigated. The flours used were commercial preparations, and in only one case did the bleached and unbleached flour come from the same batch of flour. It is not surprising, therefore, that yields of the various fractions were quite variable, precluding the drawing of any conclusions as to variation of lipid content with type or treatment of the flour. The range of values obtained, together with some typical figures, are presented in Table 4.

In preliminary studies on bleached flour lipids, it was found that the crude glycolipid mixture could be

[5] Supplied by Corn Products Corp., Pekin, Ill.
[6] See footnote 5.
[7] A gift from the Viobin Corporation, Monticello, Ill.
[8] A gift from The Quaker Oats Co., Chicago, Ill.

fractionated on a silicic-acid column, using as the eluant chloroform, with gradually increasing concentrations of methanol. The results of a typical column are shown in Table 5. A monogalactosylglycerol lipid fraction was eluted with chloroform-methanol 98/2 (v/v), followed by a digalactosylglycerol lipid fraction with increasing methanol concentrations. Obviously there is some overlapping of these main fractions, and a glycerol-containing substance is present in many of the fractions. As shown in Table 6, these fractions contained substantial amounts of chlorine, which probably accounts for some of the heterogeneity observed, since the separation obtained with a typical unbleached glycolipid sample (Table 2) was much cleaner. In both cases, however, the digalactosyl fraction showed amide absorption in the infrared. Rechromatography of the monogalactosylglycerol lipid fraction from unbleached flour gave a relatively homogeneous material free of glycerides and almost devoid of nitrogen and phosphorus. The saponification equivalent of this material (378) is in close agreement with that required for two long-chain acyl groups per molecule. Digalactosylglycerol lipid further purified by rechromatography over silicic acid gave a saponification equivalent of 466, also in close agreement with the calculated values for a diacyl derivative. The structure of these lipids is the subject of the following paper.

The infrared amide absorption of the digalactosylglycerol lipid fraction was very similar to that of a cerebroside. It was decided, therefore, to attempt the isolation of this (minor) constituent. Alkaline hydrolysis (to destroy esters) gave a crude chloroform-extractable material which, after passage over Dowex-2 (OH^-) to remove fatty acids, gave analytical data and physical properties in agreement with those for a cerebroside. The carbohydrate component proved to be glucose and the long-chain base constituent to be a mixture of three, perhaps four, components. A preliminary communication (11) has been made regarding these bases, and evidence for the structure of the cerebrosides will be presented in a subsequent paper.

In view of the relatively low solubility of cerebrosides in acetone, it seems possible that some cerebroside might have precipitated in the benzene-acetone insoluble fraction. This material, therefore, was subjected to the heptane-methanol distribution with substantial further separation of components, as shown in Table 7.

Much of the peptide nitrogen remained as insoluble interfacial material. The heptane phase contained sterol and sterol ester with little or no nitrogen or carbohydrate. The methanol phase contained (in addition to a significant amount of peptide nitrogen) the galactosylglycerol lipids and at least two different types of cerebroside, one containing glucose, the other containing mannose. Data on the separation of these two materials on silicic acid are given in Table 8. Fraction 1 (chloroform:methanol 9/1, v/v) on alkaline hydrolysis yielded the usual glucocerebroside. Fraction 2 (chloroform:methanol 8/2, v/v) gave, after alkaline hydrolysis, mannocerebroside, whose mannose content corresponded to that of a trimannoside derivative. The chloroform:methanol 6/4 (v/v) fraction from this column appeared to contain a lipopeptide.

It thus appears that wheat flour contains two galactosylglycerol lipids and a complex mixture of gluco- and mannocerebrosides, for whose isolation preparative methods are presented in this paper.

In extending these studies of the galactosylglycerol lipids to other plant sources, we have found similar materials in corn gluten, wheat germ oil, green oat groat oil, and lipids of *Euglena gracilis*, strain Z. The galactosylglycerols have been found also after hydrolysis of *Chlorella* lipids (12), and in lipids of forage grasses and clovers (13), although the lipids from these sources have not, as far as we are aware, been separated and characterized.

We are indebted to Mr. J. Nemeth and his associates from our microanalytical laboratory for these analyses.

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Wheat flour lipids: III. structure of the mono- and digalactosylglycerol lipids*

H. E. Carter, R. A. Hendry,† and N. Z. Stanacev‡

Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana, Illinois

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SUMMARY

Determination of the structures of the mono- and digalactosylglycerol lipids of wheat flour is reported. Methylation of these lipids, followed by alkaline saponification, yielded partially methylated derivatives. Periodate oxidation of these substances in each case gave 1 mole uptake with the formation of formaldehyde. These data establish the presence of two vicinal hydroxyls in the glycerol residue, and strongly indicate that in the original lipid the two acyl groups are attached to these hydroxyl groups. This assignment was confirmed by acidic hydrolysis studies. The monogalactosylglycerol derivative gave 2,3,4,6-tetra-O-methyl-D-galactose plus glycerol; the digalactosylglycerol derivative yielded, in addition, 2,3,4-tri-O-methyl-D-galactose. In a control experiment, free digalactosylglycerol was methylated and hydrolyzed, giving only on papergrams 2,3,4-tri-O-methyl- and 2,3,4,6-tetra-O-methyl-D-galactose. These results clearly define the structure of the galactosylglycerol lipids as a 2,3-diglyceride with a carbohydrate moiety attached in the 1-position. Infrared data suggest the D-configuration for the glycerol residue.

In the preceding paper (1) we described methods for the preparation from benzene extracts of wheat flour of a glycolipid fraction and its separation into mono- and digalactosylglycerol lipids on silicic-acid columns. Each of the lipids was shown to contain two fatty acid groups per molecule. The present paper reports methylation studies on these lipids leading to a complete elucidation of the structures.

EXPERIMENTAL AND RESULTS [1]

Isolation and Purification of Mono- and Digalactosylglycerol Lipids. The mono- and digalactosylglycerol lipids employed were those reported in the preceding paper (1) (the fractions 181 to 289, obtained by rechromatography of fractions 34 to 40 and 66 to 72, respectively, as shown in Table 2 of the preceding article).

Methylation of Monogalactosylglycerol Lipid. To a solution of 1.5 g of purified monogalactosylglycerol lipid in 30 ml of absolute methanol, 5.0 g of freshly prepared silver oxide and 5.0 ml of redistilled methyl iodide were added. The suspension was stirred at room temperature for 24 hours, after which time fresh portions of 5.0 g of silver oxide and 5.0 ml of methyl iodide were added. This operation was repeated twice more at intervals of 12 hours, using the same quantities of silver oxide and methyl iodide. The reaction mixture was stirred at room temperature for 48 hours after the final addition of the reagents, and then was filtered from insoluble material. The insoluble material was washed twice with methanol, and the combined methanolic solutions were evaporated to dryness *in vacuo*, giving 2.09 g of a brown viscous oil. This material was dissolved in 20 ml of absolute ether and filtered from insoluble material. The clear solution applied to 60 g of silicic acid ("Bio-Rad"; column 2.2 × 30 cm). The first fraction, eluted with ether, after evaporation of solvent, gave 1.53 g of yellow oil. The infrared spectrum of this material still showed absorption at 3600 cm^{-1}, indicating the presence of unmethylated hydroxyl groups. However, the cerebroside amide bonds at 1550 and 1650 cm^{-1}, which were present in the starting material, had com-

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† Postdoctoral Research Associate in Chemistry. Present address: Department of Chemistry, Westminster College, New Wilmington, Pa.

‡ Postdoctoral Research Associate in Chemistry.

[1] Infrared spectra were recorded with a Perkin-Elmer double beam spectrometer Model 21 with a sodium chloride prism.

pletely disappeared. Attempts to separate completely methylated from partially methylated lipid, using neutral aluminum oxide grade II, failed.

Remethylation of Monogalactosylglycerol Lipid. The partially methylated lipid was remethylated as follows, using dioxane as solvent: A suspension of 356 mg of partially methylated lipid in 30 ml of purified dioxane and 20 ml of methyl iodide was refluxed with stirring for 72 hours in the presence of 10 g of silver oxide. Fresh portions of 10 ml of methyl iodide and 10 g of silver oxide were added and the reaction mixture refluxed a further 24 hours. The hot suspension was then centrifuged, the supernatant filtered, and the yellow filtrate evaporated to dryness *in vacuo* at 45°, giving 505 mg of yellow, very viscous oil. The infrared spectrum showed complete absence of hydroxyl bonds. The paper chromatographic examination of this material did not give any permanganate-periodate or aniline-phthalate positive spots.

Alkaline Hydrolysis of Methylated Monogalactosylglycerol Lipid. A solution of 420 mg of fully methylated monogalactosylglycerol lipid in 10.0 ml of methanolic-aqueous 0.1 N sodium hydroxide was refluxed for 4 hours. After cooling at room temperature, 1.0 N hydrochloric acid was added to bring the solution to pH 1. A sufficient amount of methanol was then added to give a clear solution, which was passed through Dowex-2 (OH^-) and Amberlite®-MB-3 columns to remove fatty acids. This operation was repeated three times to achieve complete removal of the acidic material. After evaporation of solvent *in vacuo*, 193 mg of yellow oil was obtained. This oil was purified by chromatography on Whatman No. 3 filter paper in a *n*-butanol : pyridine : water 6/4/3 (v/v) solvent system. After 20 hours at room temperature (using the ascending technique), two permanganate-periodate positive substances were detected (main fraction, R_f 0.70–0.75; minor spot, R_f 0.90–0.95).

The material with R_f 0.90–0.95, in the above solvent system, gave a rather elongated spot and was eluted from the filter paper with methanol at 37° for 24 hours. Evaporation of solvent gave 38.4 mg of material, which showed a poor infrared spectrum and could not be completely characterized. The acidic acid hydrolyzate of this substance gave a spot (detected by aniline-phthalate) with an R_f similar to that of tetra-O-methyl-D-galactose. No permanganate-periodate spots were present.

The main fraction (R_f 0.70–0.75 detected by permanganate-periodate spray) was obtained by elution with methanol at 37° for 24 hours. Evaporation of the solvent gave 120.4 mg of a colorless oil. The infrared spectrum of this material showed strong absorption at 3550 cm^{-1} (hydroxyl) and the complete absence of absorption in the region of 1700 to 1750 cm^{-1}, thus establishing complete removal of ester groups. Quantitative periodate oxidation of this material will be described later.

Acidic Hydrolysis of Methylated Monogalactosylglycerol Lipid. For acidic hydrolysis, 20 to 30 mg of the R_f 0.70–0.75 material described above was used. The sample was heated with 0.5 N aqueous sulfuric acid in a sealed tube at 100° for 16 hours. The reaction mixture was neutralized with Dowex-2 (OH^-) resin, filtered free from resin, and concentrated to small volume *in vacuo*, but never to dryness. Paper chromatography, using ascending and descending techniques and Whatman No. 1 and No. 3 filter paper in two solvent systems—(*a*) *n*-butanol:pyridine:water 6/4/3 (v/v), for ascending chromatography or (*b*) ethyl acetate:acetic acid:water 3/1/3 (v/v), for descending chromatography—on the basis of at least five experiments, always showed (*a*) one intensive spot, detected with permanganate-periodate spray, with identical R_f (0.57 in ascending technique) as that of a glycerol standard; (*b*) one sharp spot, detected with aniline-phthalate spray, with same R_f (0.87 in ascending technique) as that of a 2,3,4,6-tetra-O-methyl-D-galactose standard. Co-chromatography of the unknown sample with the above standards gave similar results.

Methylation of Digalactosylglycerol Lipid. The digalactosylglycerol lipid was methylated in a similar manner to that described for the remethylation of monogalactosylglycerol lipid. A solution of 1.834 g of the lipid in 50 ml of purified dioxane was refluxed with 50 ml of methyl iodide and 25 g of silver oxide for 24 hours, after which time a new portion of 15 g silver oxide and 15 ml of methyl iodide was added and the refluxing continued for a further 24 hours. The same quantity of silver oxide and methyl iodide was again added to the reaction mixture and the refluxing continued, with stirring, for another 24 hours. After this time (72 hours of refluxing in all) the reaction mixture was worked up as described earlier, and the resultant yellow oil was purified on silicic acid. This purification is essential to remove methylation products of the contaminating cerebrosides. The partially methylated lipid was dissolved in 20 ml of ether and applied to 100 g of silicic acid ("Bio-Rad"; 2.7 × 20 cm). The first fraction, eluted with 300 ml of ether, gave a yellow oil, which still had strong absorption due to unmethylated hydroxyls, but did not show amide absorption. After two further methylations under the same conditions as described above, 1.339 g of yellow oil was obtained. This material

showed only a faint trace of infrared absorption due to hydroxyl, and on chromatographic examination was periodate-permanganate and aniline-phthalate negative.

Alkaline Hydrolysis of Methylated Digalactosylglycerol Lipid. The methylated digalactosylglycerol lipid (1.241 g) was refluxed 4 hours with 20 ml of 0.5 N methanolic-aqueous sodium hydroxide. After cooling, acidification with 1 N hydrochloric acid, and the removal of fatty acids as described—by using Dowex-2 (OH^-) and Amberlite-MB-3 column—381.5 mg of colorless oil was obtained. The infrared spectrum of this material showed strong hydroxyl absorption, but no absorption in the region of 1700 to 1750 cm^{-1}. This oil was further purified either by using preparative paper chromatography, as described for the methylated monogalactosylglycerol, or, more efficiently, by using a carbon:celite column.

For purification on a carbon:celite (1:1) column, 100 mg of the alkaline hydrolysis product was dissolved in 5 ml of 80% ethanol and applied to the column (1.8 × 7 cm), prepared by the procedure described earlier (2). Fraction 1, eluted with 50 ml of 80% ethanol, gave, after evaporation of solvent, 74 mg of colorless oil. This material, on paper chromatography, was permanganate-periodate positive and aniline-phthalate negative, and showed strong absorption in the infrared at 3500 cm^{-1} (hydroxyl) with no absorption in the region of 1700 to 1750 cm^{-1}. The absorption due to amide bonds (cerebrosides) also disappeared. Quantitative periodate study of this substance will be described later.

Acidic Hydrolysis of Methylated Digalactosylglycerol Lipid. The acidic hydrolysis of deacylated methylated derivative was performed in the same manner as described above, using 0.5 N aqueous sulfuric acid in a sealed tube for 16 hours at 100°. Paper chromatography, using the same techniques and solvent systems as described for methylated monogalactosylglycerol, showed (*a*) one intensive spot, detected with permanganate-periodate spray, with the same R_f (0.57 in ascending technique) as glycerol standard; (*b*) two spots, detected with aniline-phthalate spray, with the same R_f as standards of 2,3,4-tri-O-methyl-D-galactose (0.78 in ascending technique), and 2,3,4,6-tetra-O-methyl-D-galactose (0.87 in ascending technique). Co-chromatography of the unknown sample with above standards gave similar results, indicating the identity of the unknown mixture with the applied standards of glycerol, 2,3,4-tri-O-methyl- and 2,3,4,6-tetra-O-methyl-D-galactose.

Methylation of Digalactosylglycerol. Digalactosylglycerol, obtained by alkaline hydrolysis of lipid, as described above, was methylated by the procedure of Kuhn *et al.* (3). To a solution of 350 mg of carbohydrate in 10 ml of redistilled dimethylformamide, 6.8 g of methyl iodide and 3.2 g of silver oxide were added. The reaction mixture was shaken 16 hours in a stoppered glass cylinder. The contents of the cylinder were then transferred to a centrifuge tube and centrifuged. The supernatant, combined with the washings (dimethylformamide and chloroform), was washed with a solution of 0.4 g of potassium cyanide in 60 ml of water. The dimethylformamide-chloroform layer was separated, and the aqueous solution extracted five times with 10 ml of chloroform. The combined extracts, after washing four times with 25 ml of water, were dried over sodium sulfate and evaporated to dryness. The residual syrup was purified by distillation at 280° to 290° *in vacuo* (1 to 2 mm of Hg). A yield of 158 mg of colorless oil (or 36.5% of theory) of the nona-O-methyl-digalactosylglycerol was obtained. The analysis was in agreement with calculated values.

	$C_{24}H_{46}O_{13}$ (542.61)
Calculated:	C 53.11; H 8.55
Found:	C 53.22; H 8.61

This oil, after several weeks, crystallized to a white solid with m.p. 52°–54°. Infrared analysis showed only a faint trace of absorption due to hydroxyl group.

The methylated digalactosylglycerol (10 mg) was hydrolyzed with acid in the usual way. The hydrolyzate, after neutralization with Dowex-2 (HCO^-_3), was analyzed by the described paper chromatographic techniques. The solvent system was the upper layer of *n*-butanol:ethanol:water:ammonia, 40/10/49/1 (v/v). Only two spots were detected with 5% ammoniacal silver nitrate spray: one corresponded to 2,3,4,6-tetra-O-methyl-D-galactose (R_f 0.80) and the other to 2,3,4-tri-O-methyl-D-galactose (R_f 0.60). No glycerol was found in the hydrolyzate, although a strong spot was obtained with the standard (R_f 0.41). This indicated that the glycerol moiety had been methylated and was released on acid hydrolysis as 2,3-di-O-methylglycerol, which would not be expected to react with the spray employed

Periodate Oxidation Studies. Oxidation of Deacylated Methylated Monogalactosylglycerol Lipid (Tetra-O-methylgalactosylglycerol). The method used for these studies was described by Reeves (4). To a solution of 16.2 mg of deacylated methylated monogalactosylglycerol lipid, in 2 ml of absolute methanol, 1 ml of a 0.2 M aqueous solution of sodium metaperiodate was added. The reaction mixture was left at room temperature for 12

hours in the dark, after which time an aliquot was taken for a determination of periodate consumption. The estimated consumption of sodium periodate was 1.03 mole per mole of methylated monogalactosylglycerol.

The main part of the reaction mixture was reduced with 4 ml of 0.2 M disodium arsenite solution at room temperature for 3 hours, and the reaction mixture then buffered with 2 ml of 1 N sodium acetate:1 N hydrochloric acid 1/1 (v/v) solution. One milliliter of 5% ethanolic solution of dimedon was added, the reaction mixture was heated to reflux for 2 to 3 minutes, and then cooled at 4° for 6 hours. A dimedon derivative of formaldehyde (6.2 mg) with m.p. 189.5°–190.5° was obtained in yield of 43% of theory.

Oxidation of Deacylated Methylated Digalactosylglycerol Lipid (Hepta-O-methyl-digalactosylglycerol). To the solution of 32.9 mg of deacylated methylated digalactosylglycerol lipid in 10 ml of absolute methanol, 5 ml of 0.2 M aqueous sodium periodate was added and the reaction mixture left for 12 hours at room temperature. An aliquot was removed for determination of periodate consumption. The estimated consumption of sodium periodate was 1.34 mole per mole of methylated digalactosylglycerol. The remaining reaction mixture was reduced with 25 ml of 0.2 M sodium arsenite for 4 hours at room temperaure, buffered with 20 ml of 1 N sodium acetate:1 N hydrochloric acid 1/1 (v/v), and to this solution 10 ml of 5% dimedon in absolute ethanol was added. The clear reaction mixture was heated to boiling for 2 to 3 minutes, cooled at 4° for 6 hours, and filtered, giving 10.4 mg of white crystals with m.p. 183°–185°. After one crystallization from aqueous methanol, this material gave 9.0 mg (55% of theory) of the dimedon derivative of formaldehyde with m.p. 190.5°–191.5°.

Determination of the Configuration of the Glycerol Residue in Monogalactosylglycerol Lipid. The alkaline hydrolysis of monogalactosylglycerol lipid gave an aqueous solution which was deionized by passing through Amberlite®-MB-3 resin and then lyophilized. The residue gave crystalline monogalactosylglycerol (m.p. 139.5°–142°, $[\alpha]^{27} = +3.77°$), as described previously (2). The infrared spectrum of this substance (on KBr) was completely identical in the fingerprint region with that of the O-β-galactopyranosyl-(1 → 1)-D-glyceritol (m.p. 140.5°–141.5°, $[\alpha]^{20} = -7°$), synthesized by Wickberg (5). On the basis of this evidence it is concluded that the glycerol residue in the original lipid has the D configuration. Although the natural and synthetic compounds have similar melting points and superimposable infrared spectra, they differ somewhat in rotations, which remains to be explained. A similar conclusion has been reached by Wickberg (6), employing a sample of our monogalactosylglycerol from wheat flour lipid.

DISCUSSION

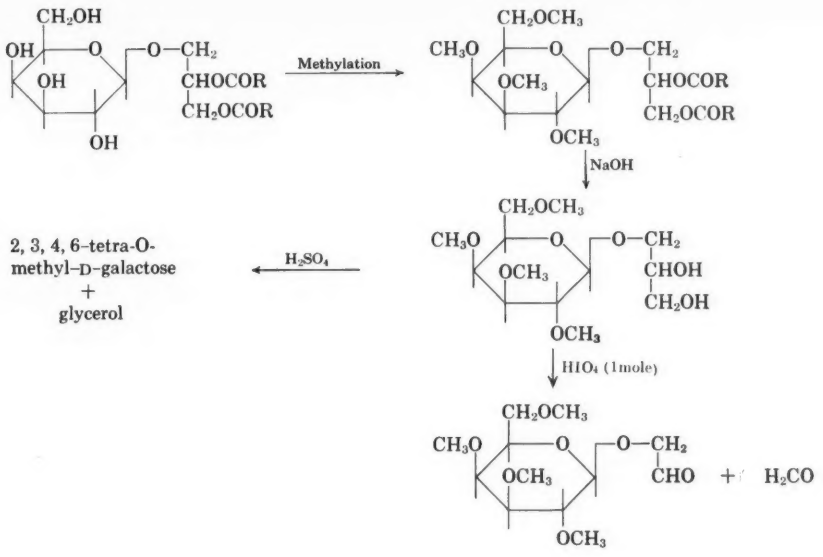
In 1956 we reported (2) fractionation studies on the benzene-extractable lipids of wheat flour, and described the partial separation of two galactosylglyceride components. Two problems arose from this study. The first concerned the identification of the galactosylglycerol moiety, obtained by alkaline hydrolysis of both lipids; the second concerned the structure of the intact lipids. In the initial communication (2) the carbohydrate moieties (obtained by alkaline hydrolysis of the lipids) were characterized as β-D-galactopyranosyl-1-glycerol, and α-D-galactopyranosyl-1,6-β-D-galactopyranosyl-1-glycerol. The second paper in this series (1) described the quantitative determination of the products of hydrolysis of both lipids, and the semiquantitative determination of the composition of the fatty acids. On the basis of these studies it was concluded that in both mono- and digalactosylglycerol lipids, 2 moles of fatty acids were present per mole of lipid. This fact should be noted, since a galactosylglycerol lipid from *Chlorella* was reported (7, 8) to have a monoglyceride structure. In the case of wheat flour glycolipids, final proof of the presence of 2 moles of fatty acids per mole of glycolipids is provided by the experiments reported in this paper.

Methylation, followed by alkaline deacylation, gave, in the case of both glycolipids, products that consumed 1 mole of periodate per mole with the formation of formaldehyde. Acidic hydrolysis of these products released only 2,3,4-tri-O-methyl-D-galactose and unmethylated glycerol, and 2,3,4-tri-O-methyl-D-galactose, 2,3,4,6-tetra-O-methyl-D-galactose and unmethylated glycerol, respectively. This evidence demands a structure with two vicinal esterified hydroxyls in the glycerol moiety of the lipid, i.e., the 2,3-diacyl-glyceryl-glycoside structure.

The reactions involved with monogalactosylglycerol lipid are shown in the equations on page 227.

It should be noted that the structure previously assigned to the digalactosylglycerol moiety is confirmed by the methylation studies, since the methylation product on acid hydrolysis gave essentially equimolar quantities of 2,3,4-tri-O-methyl- and 2,3,4,6-tetra-O-methyl-D-galactose.

Wickberg has isolated and characterized mono- and digalactosylglycerol from red algae, and for comparative studies has synthesized a series of 1-glycerol α- and β-galactosides, in both the D and L forms (5, 6). These synthetic glycosides have characteristic and



different absorptions in the frequency range 730 to 960 cm^{-1} (5). On the basis of physical properties and infrared spectra of the material isolated from red algae and synthetic samples, Wickberg was able to assign the D configuration to digalactosylglycerol from red algae, and to show its identity with our digalactosylglycerol from wheat flour. However, the monogalactosylglycerol isolated from red algae was found to be an isomorphous mixture of the D- and L-glycerol derivatives. Our monogalactosylglycerol, isolated from wheat flour, has an infrared spectrum identical with synthetic O-β-D-galactopyranosyl-D-glycerol, and therefore the glycerol residue has the D configuration. These data establish the D configuration of the glycerol residue in each of the galactosylglycerol lipids isolated from wheat flour.

In view of these experimental data, we assign to the monogalactosylglycerol lipid, isolated from unbleached wheat flour, the structure of 2,3-diacyl-1-β-D-galactopyranosyl-D-glycerol, and to the digalactosylglycerol lipid isolated from the same source, the structure of 2,3-diacyl-1-(α-D-galactopyranosyl-1,6-β-D-galactopyranosyl)-D-glycerol, with the gross composition of fatty acids described earlier (1).

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Biochemistry of the sphingolipids: XII. conversion of cerebrosides to ceramides and sphingosine; structure of Gaucher cerebroside*

H. E. Carter, J. A. Rothfus,† and Roy Gigg‡

Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana, Illinois

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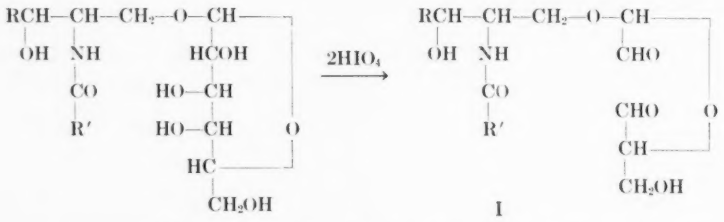
SUMMARY

An improved procedure for the conversion of cerebroside to ceramide and sphingosine has been devised, consisting of periodate opening of the glycosidic ring, reduction with $NaBH_4$, and hydrolysis under very mild acid conditions to ceramide. The over-all yield is excellent and only the erythro isomer results. Alkaline hydrolysis of ceramide gives erythro-sphingosine in good yield. Application of this procedure to Gaucher cerebroside has shown it to contain the "normal" erythro-sphingosine.

Cerebrosides are readily available in quantity and would provide a convenient source of ceramide and sphingosine if a satisfactory procedure for removing the galactosyl moiety were available. Unfortunately, cerebrosides are moderately resistant to acid hydrolysis and, under the vigorous conditions required, yield not only "natural" erythro-sphingosine but also the threo isomer (1, 2, 3) (in aqueous solution), the 3-O-methyl ethers (4) (with methanolic acids), and other degradation products. An effective method for cleavage of cerebrosides to ceramides and sphingosine without stereochemical alterations would be valuable both for preparative purposes and for elucidation of the stereochemistry of cerebrosides.

To this end, therefore, a study of the periodate oxidation of cerebrosides was undertaken in the hope that destruction of the glycosidic ring structure would labilize the acetal bond.



I

A chloroform-ethanol-water system was found to be a convenient solvent for this reaction since it dissolves both cerebroside and periodic acid, with a resultant rapid, clean-cut uptake of 2 moles of periodate. The "dialdehyde" product can be isolated in excellent yield as a white, waxy solid giving acceptable analytical data. However, attempts to cleave this material under mild conditions (acidic or alkaline) were unsuccessful. Treatment of the "dialdehyde" with phenylhydrazine or cyclohexylamine (modified procedure of Barry and Mitchell [5]) gave poor yields of ceramide contaminated with nitrogenous impurities whose separation proved difficult.

The acid stability of periodate-oxidized cerebrosides is similar to that displayed by periodate oxidation products of other glycosides (6). Numerous studies have shown that these "dialdehydes" form cyclic structures (often with the inclusion of a molecule of water)

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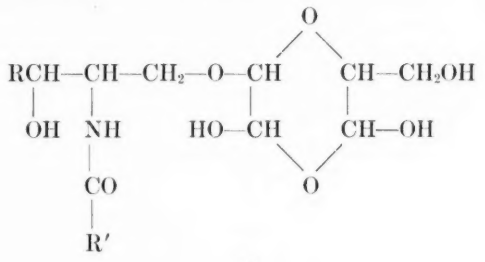
† Present address: University of Utah School of Medicine, Salt Lake City 12, Utah.

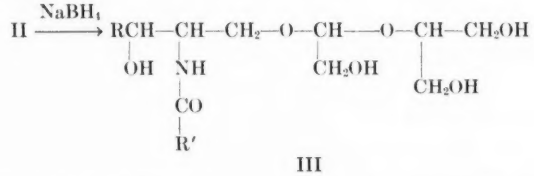
‡ Present address: National Institute for Medical Research, Mill Hill, London, N.W. 7, England.

(7 to 15), which may account in part for their stability. That the cerebroside "dialdehyde" also exists in a cyclic form (II rather than I) is indicated by the absence of an aldehyde carbonyl band in the infrared spectrum. Also, the analytical data indicate the presence of an additional molecule of water.



II

The periodate-oxidized cerebroside was readily reduced to the polyol (III) by sodium borohydride (for preparative purposes it is unnecessary to isolate the "dialdehyde"). The reduction products were white solids whose analytical data supported the assigned structure



III

Hydrolysis of III with very mild acid gave ceramide in excellent yield, and alkaline hydrolysis of the latter gave erythro-sphingosine in excellent over-all yields (up to 85%) with no evidence for the production of the threo isomer. Thus these reactions provide a satisfactory preparative method for converting cerebroside to ceramide and sphingosine.

Recent reports on the structure of Gaucher cerebroside have shown that glucose is the sole carbohydrate component (16, 17); that it is attached to carbon-1 of sphingosine (17); and that the acid moiety consists mainly of a mixture of palmitic, behenic, and lignoceric acids (17). However, the stereochemistry of the sphingosine constituent and of the glycosidic bond to sphingosine has not been established. It seemed possible that the accumulation of cerebroside in Gaucher spleen might be due to the abnormal formation of the threo epimer, particularly in view of the activity of threo-sphingosine derivatives as biosynthetic precursors of sphingolipids (18, 19). Therefore a comparative study was made of the degradation of kerasin and Gaucher spleen cerebroside by the above procedure. Both cerebrosides contain normal saturated acid moieties (lignoceric in kerasin) and differ in that kerasin contains galactose as the carbohydrate constituent. If no other stereochemical difference existed, opening of the glycosidic ring should yield identical products from the two substances except for possible homologous variation in acid chain length (see structures I, II, and III). A comparison of the "dialdehyde" and "polyol" from kerasin and Gaucher cerebroside shows such close similarities that there can be no doubt as to their stereochemical identity. Furthermore, Gaucher ceramide yielded only erythro-sphingosine on further hydrolysis. These data establish that Gaucher cerebroside has the normal sphingosine structure and, furthermore, that the glucosyl moiety has the same anomeric configuration as the galactosyl constituent of kerasin. This finding is in agreement with the results of Rosenberg and Chargaff (16), based on spectroscopic evidence, that both normal and abnormal cerebrosides have the β-configuration of the glycosidic group.

EXPERIMENTAL

Preparation of Cerebrosides. Phrenosin and kerasin were obtained from a crude mixture of sphingolipids generously provided by the Wilson Co., Chicago, Illinois. Alkali-labile phospholipids and plasmalogens were first removed from the mixture (20), and the residue was then chromatographed on silicic acid-Celite®. A column 7 cm in diameter and 70 cm high was filled with a chloroform slurry of 1000 g of silicic acid (Mallinckrodt, analytical reagent, 100 mesh) and 350 g of Celite®. All bubbles of occluded air were removed, and the slurry was allowed to pack by gravity. The column was then washed with 2200 ml of methanol followed by an equal volume of chloroform. The sample (19.9 g) was dissolved in a minimum amount of hot chloroform-methanol 9/1 (v/v), and the solution, while still warm, was applied to the column. As the solution cooled and began to pass through the column, a layer of lipid was deposited in the first 2 cm of the column. One liter of chloroform-methanol 9/1 (v/v) was then applied to the column, followed by 3 liters of chloroform-methanol 8/2 (v/v). Subsequent elution was carried out with increasing amounts of methanol in chloroform. Since the first fractions eluted contained some colored matter, it was possible to complete their separation by collecting fractions manually. A crude kerasin fraction (3.2 g) followed by a crude phrenosin fraction (8.2 g) were eluted with chloroform-methanol 8/2 (v/v).

Purification of the phrenosin was accomplished by allowing a saturated solution of the crude material in low boiling petroleum ether-methanol 10/1 (v/v) to

percolate through a column of 40 g of Merck alumina which had been neutralized, washed, and dried for 24 hours at 110°. Evaporation of the effluent solution resulted in the recovery of 4.1 g of a light tan powder (impure phrenosin). White finely powdered phrenosin (2.15 g) was obtained by washing the column with a mixture of low boiling petroleum ether and methanol 1/1 (v/v). A final 0.53 g was recovered with chloroform-methanol-water 2/2/1 (v/v). The petroleum ether-methanol 1/1 (v/v) fraction was essentially pure phrenosin.

$C_{48}H_{93}NO_9$ (828.23)
Calculated: C 69.60, H 11.32, N 1.69
Found: C 67.91, H 11.17, N 1.63
$[\alpha]_D^{30} = +2.47°$ (4.86% in dry pyridine)

Hexa-acetylphrenosin was prepared from this product with acetic anhydride in pyridine (21). (Saponification equivalent: calculated 180; found 178. $[\alpha]_D^{32} = -7.32°$ [6.78% in chloroform-methanol 1/1, v/v].)

The crude kerasin was purified by recrystallization from chloroform-methanol 2/8 (v/v) followed by rechromatography on silicic acid-Celite®. A column 2 cm in diameter and 44 cm high was prepared as described above. The sample was applied as a solution in 50 ml of chloroform-methanol 95/5 (v/v). The column was first washed with 225 ml of chloroform; then chloroform-methanol 8/2 (v/v) was used to elute the pure cerebroside.

$C_{48}H_{93}NO_8$ (812.23)
Calculated: C 70.98, H 11.54, N 1.72
Found: C 70.44, H 11.05, N 1.75
$[\alpha]_D^{29} = +2.48°$ (4.89% in chloroform-methanol 1/1, v/v)
$[\alpha]_D^{30} = -2.95°$ (4.89% in dry pyridine)

An acetylated derivative of this material exhibited the following properties: Saponification equivalent: calculated 204; found 202. $[\alpha]_D^{32} = -12.9$ (7.25% in chloroform-methanol 1/1, v/v).

Gaucher cerebroside was prepared directly from a segment of a Gaucher spleen, which was made available through the generosity of Dr. R. O. Brady, of the National Institutes of Health, Bethesda, Maryland. The spleen segment was stored in the frozen state or at 4° under acetone during 20 months which passed between the time it was removed and the time at which these experiments were undertaken.

The acetone-saturated tissue (320 g) was blended, in two batches, in a Waring Blendor for 5 minutes at room temperature with a total of 1 liter of acetone. The blended mixture was allowed to stand at 4° for 4 hours and then was filtered by suction. The collected solid was suspended in 500 ml of dry acetone and stored at 4° for an additional 12 hours. At the end of this time, the solid was separated by filtration and again suspended in 500 ml of dry acetone. After standing at room temperature for 2 hours, the mixture was filtered, giving 272 g of acetone-moist tissue.

The cold acetone-extracted tissue was then subjected to continuous extraction with hot acetone in a Soxhlet apparatus for 40 hours. When cooled to room temperature, the extract yielded a light tan precipitate which was collected by centrifugation, washed four times with 15-ml portions of water, twice with acetone, and dried. The crude product weighed 10.5 g and gave an infrared spectrum which was devoid of ester absorption.

The material was further purified by chromatography on silicic acid-Celite®. A column 3.5 cm in diameter and 45 cm high was prepared according to the method described above. The sample (3.42 g) was applied to the column as a solution in 50 ml of chloroform-methanol 5/1 (v/v). One column volume (500 ml) of reagent chloroform was sufficient to remove a small amount of colored matter and all of the cerebroside (3.11 g). The purified cerebroside was a white powder which melted at 185°–187°.

$C_{46}H_{89}NO_8$ (784.18)
Calculated:[1] C 70.45, H 11.44, N 1.79
Found: C 70.27, H 11.04, N 1.71
$[\alpha]_D^{29} = -5.58°$ (5.34% in chloroform-methanol 1/1, v/v)
$[\alpha]_D^{30} = -8.94°$ (5.34% in dry pyridine)

When acetylated in the usual manner, the Gaucher cerebroside gave a derivative which melted at 94°–96°. Analytical data were in good agreement with those of a penta-acetyl derivative.

$C_{56}H_{99}NO_{13}$ (994.36)
Calculated: C 67.64, H 10.04, N 1.41, S.E., 199
Found: C 67.88, H 10.05, N 1.59, S.E., 206
$[\alpha]_D^{28} = -19.65°$ (5.9% in chloroform-methanol 1/1, v/v)
$[\alpha]_D^{28} = -25.09°$ (5.9% in dry pyridine)

Periodate Oxidation of Cerebroside. Finely powdered cerebroside (1 g) was transferred to a 100-ml volumetric flask and suspended in a mixture of 14 ml of chloroform and 51 ml of 95% ethanol. Oxidation was initiated by the addition of a measured amount (30.27 ml) of 0.2 M aqueous periodic acid. The mixture was diluted to volume with ethanol, shaken vigorously until all the lipid had dissolved, and was then allowed to stand at room temperature in the dark. Periodically, 3-ml aliquots were withdrawn from the oxidation mixture and titrated (22) against an aliquot from a blank reaction mixture to determine the extent of oxidation

[1] For purposes of comparison, the average chain length of the fatty acid constituents of the Gaucher cerebroside was assumed to be C_{22}.

(Table 1). Upon completion of the oxidation, excess reagent was reduced by the addition of 1 ml of ethylene glycol.

TABLE 1. PERIODATE OXIDATION OF CEREBROSIDES

Time	Periodic Acid Reduced	
	Kerasin	Gaucher Cerebroside
hours	*moles/mole*	*moles/mole*
1	2.03	1.35
2	1.99	1.75
3	1.99	
4		1.93
5		1.94

Although the combination of solvents used resulted in a homogenous system in which both the cerebroside and oxidizing agent remained soluble throughout the course of the reaction, any large variation in composition was sufficient to cause the mixture to separate into two phases. Thus, to recover the product, the oxidation mixture was poured into an equal volume of water; and the chloroform layer which formed was withdrawn, washed, and evaporated. Additional traces of product were obtained by further extraction of the diluted mixture with chloroform. Removal of the solvent gave the crude periodate oxidation product as a white, waxy solid.

Periodate-oxidized phrenosin: m.p. 131°–133°; $[\alpha]_D^{25} = -17.95°$ (2.95% in chloroform)

$C_{47}H_{91}NO_9$ (814.21)

Calculated: C 69.33, H 11.26, N 1.72

Found: C 68.86, H 11.09, N 1.88

Periodate-oxidized kerasin: m.p. 83°–88°

Periodate-oxidized Gaucher cerebroside: m.p. 88°–96°

An infrared spectrum of periodate-oxidized phrenosin was devoid of aldehydic carbonyl absorption indicating the existence of a cyclic "hemiacetal" structure.

Attempts to Hydrolyze Oxidized Cerebrosides with Acid and Alkali. Treatment of periodate-oxidized phrenosin (121 mg) at 37° with 0.12 N HCl in methanol for 10 hours followed by 3 hours with 0.12 N HCl in 50% aqueous methanol gave a chloroform extractable ninhydrin-negative product (117 mg), which melted in the range for known ceramides (m.p. 78°–81°) after recrystallization from acetone (C 69.58, H 11.30, N 1.85; $[\alpha]_D^{24} = -15.95°$ [2.15% in chloroform]). This product was, however, strongly aniline phthalate-positive and in other ways appeared to be practically identical to the starting material. A similar product (m.p. 80°–85°; $[\alpha]_D^{27} = -12.60°$) was obtained by refluxing periodate-oxidized phrenosin with 0.1 N HCl in 70% aqueous dioxane for 3 hours.

Attempts to obtain a ceramide or sphingosine from oxidized cerebrosides by alkaline hydrolysis were equally unsuccessful. Periodate-oxidized phrenosin (154 mg) was refluxed in 30 ml of 0.01 N NaOH in 70% aqueous dioxane for 6.5 hours in a carbon dioxide-free system. The mixture was cooled, diluted with an equal volume of water, and extracted quickly with chloroform. The extract yielded 143 mg of a white powder (m.p. 178°–182°) which gave no reaction with either aniline phthalate or ninhydrin (C 66.42, H 10.74, N 1.83 [alkaline residue]).

Periodate-oxidized phrenosin (218 mg) was also refluxed for 14 hours in 14 ml of one-quarter saturated $Ba(OH)_2$ in 50% aqueous dioxane. In this case the reaction mixture was neutralized before extraction with chloroform. Enough methanol was present during the extraction to prevent the formation of emulsions. Upon evaporation of the extracts, it was found that approximately half of the material was then chloroform-methanol insoluble and high melting (m.p. 220°). The remainder melted from 85° to 110° and was ninhydrin-positive but did not yield triacetylsphingosine when treated with acetic anhydride in pyridine.

Degradation of Periodate-Oxidized Cerebrosides with Cyclohexylamine. A modification of the method of Barry and Mitchell (5) for degrading periodate-oxidized polysaccharides was used as a possible means of obtaining crude ceramides from oxidized cerebrosides.

Periodate-oxidized phrenosin (500 mg) was suspended in a solution composed of 5 ml of cyclohexylamine and 50 ml of methanol, and the mixture was refluxed for 2 hours. Thereupon the methanol was removed under reduced pressure, and the residue was suspended in approximately 50 ml of water. This mixture was cooled on ice, slowly acidified to pH 1 with 6 N H_2SO_4, and allowed to stand at room temperature for 2 hours. The lipid was then extracted into chloroform. Enough methanol was added to prevent the formation of emulsions. The extracts were neutralized with Dowex 2 (HCO_3^-), filtered, and evaporated to dryness. The residue obtained was extracted exhaustively with hot acetone, and the extracts were concentrated, then cooled to room temperature to precipitate the crude ceramide (m.p. 78°–88°). After recrystallization from acetone, the product was a slightly tan powder (m.p. 83°–89°).

$C_{42}H_{83}NO_4$ (666.09)

Calculated: C 75.73, H 12.56, N 2.10

Found: C 73.09, H 11.95, N 3.00

This product was hydrolyzed with 1 N KOH in 90% methanol according to the method described in a following section. Acetylation of the liberated base gave crude triacetylsphingosine. Two recrystallizations from *n*-hexane yielded the pure derivative as fine white needles (m.p. 104°–106°; $[\alpha]_D^{27} = -10.1°$ [1.43% in chloroform]).

	$C_{24}H_{43}NO_5$ (425.59)
Calculated:	C 67.73, H 10.18, N 3.29
Found:	C 67.56, H 10.37, N 3.74

The yields of the products obtained by this method are summarized in Table 2. Since contaminating substances encountered with this method were also soluble in organic solvents, efficient purification of the products was difficult to accomplish by simple methods.

TABLE 2. CYCLOHEXYLAMINE DEGRADATION OF PHRENOSIN

Compound	Over-all Yield from Phrenosin
	per cent
Oxidized phrenosin	98.9
N-Cerebronylsphingosine (crude)	78.8
N-Cerebronylsphingosine (purified)	64.5
Sphingosine	64.5
Triacetylsphingosine (crude)	59.5
Triacetylsphingosine (purified)	29.3

Reduction of Periodate-Oxidized Cerebrosides. A more efficient method of degrading oxidized cerebroside was designed to take advantage of the increase in lability which periodate-oxidized glycosides exhibit upon reduction to the corresponding alcohols (23). Very finely powdered oxidized cerebroside (960 mg) was dissolved in 13 ml of chloroform. The solution was diluted with 56 ml of 95% ethanol and 20 ml of water, and then was rendered basic by the addition of 4 drops of 20% NaOH. A solution of 100 mg (fourfold excess) of $NaBH_4$ in 10 ml of 0.1 N NaOH was added with stirring, and the reaction was allowed to proceed at room temperature for 3 hours. The solution was stirred continuously and remained clear throughout this period. Reduction was terminated by careful acidification of the reaction mixture to pH 1 with methanolic HCl to decompose the excess reagent. The solution was then neutralized with 20% NaOH and poured into an equal volume of water. After standing overnight, the product-containing chloroform layer was siphoned off, and the reaction mixture was extracted twice more with chloroform. These extracts were washed twice with water and evaporated under reduced pressure to obtain the product.

Phrenosin derivative: m.p. 148°–151° (melted and resolidified, 88°–105°; $[\alpha]_D^{25} = +20.3°$ [1.42% in chloroform-methanol 1/1, v/v]).

Acetylated phrenosin derivative: m.p. 58°–60°; $[\alpha]_D^{26} = +0.226°$ (3.10% in chloroform-methanol).

	$C_{57}H_{103}NO_{13}$ (1010.40)
Calculated:	C 67.75, H 10.28, N 1.39, S.E. 202
Found:	C 67.31, H 10.23, N 1.50, S.E. 200

Kerasin derivative: m.p. 113°–118° (sintered circa 75°); $[\alpha]_D^{32} = +5.90°$ (5.13% in chloroform-methanol 1/1, v/v); $[\alpha]_D^{32} = -0.80°$ (5.09% in dry pyridine).

Acetylated kerasin derivative: m.p. 63°–65°; saponification equivalent: calculated 238; found 234.

Gaucher cerebroside derivative: m.p. 111°–114° (sintered circa 75°); $[\alpha]_D^{32} = +5.70°$ (5.14% in chloroform-methanol 1/1, v/v); $[\alpha]_D^{32} = -0.78°$ (5.08% in dry pyridine).

Acetylated Gaucher cerebroside derivative: m.p. 70°–73°; saponification equivalent: calculated 231; found 236.

Reduction of the double bond in chemically reduced periodate-oxidized kerasin and Gaucher cerebroside was effected by shaking 1% solutions of the lipids in warm absolute ethanol in an Adams apparatus for 10 hours at room temperature and 2 atmospheres of pressure in the presence of 80 mg of platinic oxide (J. Bishop and Co., Malvern, Pa.).

Kerasin derivative: m.p. 124°–129° (melted and resolidified, 85°–105°); $[\alpha]_D^{32} = +34.5°$ (5.04% in chloroform-methanol 1/1, v/v).

Gaucher cerebroside derivative: m.p. 115°–121° (melted and resolidified, 85°–105°); $[\alpha]_D^{32} = +33.5°$ (4.98% in chloroform-methanol 1/1, v/v).

Hydrolysis of Reduced Periodate-Oxidized Cerebrosides. The reduction product (830 mg) was dissolved in 60 ml of 0.1 N HCl in 90% aqueous peroxide-free tetrahydrofuran. This solution was then allowed to stand at room temperature for 24 hours. The length of the hydrolysis period was established by following the reaction optically to constant rotation. After hydrolysis, the mixture was diluted with an equal volume of water, neutralized with solid Na_2CO_3, and extracted three times with chloroform. The extracts were combined, washed twice with equal volumes of water, and then evaporated to dryness to obtain the crude product. This material was dissolved in 100 ml of hot absolute methanol and filtered to remove a small amount of an insoluble brown wax. The filtrate was concentrated under reduced pressure to approximately

25 ml, heated to dissolve any solid, then cooled to 0° to reprecipitate a white powder.

Phrenosin ceramide (cerebronyl sphingosine): m.p. 82°–86°.

	$C_{42}H_{83}NO_4$ (666.09)
Calculated:	C 75.73, H 12.56, N 2.10
Found:	C 72.60, H 11.99, N 2.30

Kerasin dihydroceramide (lignoceryl dihydrosphingosine): m.p. 95°–98°; $[\alpha]_D^{32}$ = +4.50° (2.51% in chloroform-methanol 1/1, v/v).

	$C_{42}H_{85}NO_3$ (652.11)
Calculated:	C 77.35, H 13.14, N 2.15
Found:	C 76.66, H 12.50, N 2.03

Gaucher cerebroside dihydroceramide: m.p. 94°–97°; $[\alpha]_D^{32}$ = +4.40° (2.48% in chloroform-methanol 9/1, v/v).

	$C_{40}H_{81}NO_3$ (624.06)
Calculated:	C 76.98, H 13.08, N 2.24
Found:	C 76.41, H 12.56, N 2.29

Infrared spectra of these materials showed only the absorption maxima expected of ceramides.

The aqueous solution (150 ml) obtained from the hydrolysis of the Gaucher material was filtered to remove a slight white precipitate and then was treated with a solution of 1.5 g of phenylhydrazine hydrochloride and 3 g of sodium acetate in a minimum amount of water. Within 5 minutes there was formed a bright yellow precipitate which slowly turned brown-orange upon standing at room temperature. After 15 hours the solution was filtered to obtain 91 mg of crude glyoxal phenylosazone (m.p. 156°–161°). Recrystallization from aqueous ethanol gave fine golden needles (m.p. 158°–165°).

	$C_{14}H_{14}N_4$ (238.28)
Calculated:	C 70.56, H 5.92, N 23.52
Found:	C 71.05, H 5.77, N 22.49

In contrast to the ease with which the reduced cerebroside derivatives could be cleaved with acid, treatment with 0.1 N NaOH in 70% dioxane at reflux for 6 hours gave little or no degradation.

Preparation of Ceramide from Phrenosin. For preparative purposes the three steps (periodate oxidation, $NaBH_4$ reduction, and acid hydrolysis) can be carried out without isolation of intermediate products as follows.

A sample of 1.00 g of the finely powdered colorless cerebroside (1.21 mmoles as phrenosin) was transferred to a 100-ml volumetric flask and suspended in a mixture of 14 ml of chloroform and 51 ml of 95% ethanol. Oxidation was initiated by the addition of a measured amount (30.4 ml) of 0.2 M aqueous periodic acid. The mixture was diluted to volume with ethanol, shaken vigorously until all the lipid had dissolved, and was allowed to stand at room temperature in the dark for 3 hours.

After reduction of excess periodic acid by the addition of 1 ml of ethylene glycol, 80 ml of water was added to the reaction mixture and the chloroform layer which formed was withdrawn. The water layer was extracted successively with 22-ml and 10-ml portions of chloroform to obtain additional small amounts of product. The combined chloroform extracts were then washed with 20 ml of water and with a mixture of 18 ml of methanol and 20 ml of water.

The washed chloroform extract (52 ml) was transferred to a 500-ml flask, and 224 ml of 95% ethanol and 80 ml of water were added. The solution was neutralized to pH 9 with several drops of 20% aqueous NaOH and a solution of 100 mg (2.64 mmoles) of $NaBH_4$ in 40 ml of 0.1 N NaOH was added dropwise from a pipette over a period of several minutes. The flask was then stoppered loosely and stirring was continued at room temperature for 6 hours. Reduction was terminated by careful neutralization of the reaction mixture to pH 6.8 with aqueous 6 N HCl.

The neutralized solution was further acidified with 6.7 ml of aqueous 6 N HCl in order to make the final HCl concentration of the solution 0.1 N. The solution was allowed to stand at room temperature for 24 hours. During this period the solution remained clear and colorless. The reaction mixture was diluted with water and repeatedly extracted with a mixture of chloroform and methanol 1/1 (v/v). These extracts were combined, washed with water several times, and evaporated to dryness, giving 744 mg (93% yield) of a colorless powder (m.p. 92°–94°) which was almost pure ceramide (C 75.15, H 12.34, N 2.20).

Isolation of Free Bases. Two methods were used to hydrolyze the ceramides to sphingosine (or dihydrosphingosine).

The phrenosin ceramide was refluxed for 10 hours at a concentration of 1.2% in a solution of 1 N KOH in 90% methanol. The hydrolysis mixture was diluted with an equal volume of water, allowed to stand for 12 hours at room temperature, and then extracted three times with equal volumes of ether. During the extraction enough methanol was maintained in the mixture to prevent the normally ether-insoluble base from precipitating. The ether extracts were washed until neutral, then were evaporated to recover the crude product.

The dihydroceramides from kerasin and Gaucher cerebroside were each hydrolyzed at a concentration of 0.8% in solutions consisting of peroxide-free dioxane

(50%), carbon dioxide-free water (25%), and aqueous saturated $Ba(OH)_2$ (25%). The mixtures were refluxed for 24 hours with occasional stirring to dislodge any materials which collected on the sides of the flasks. At the end of this time 3 volumes of water were added, and the solutions were filtered with suction to obtain white powders from which crude dihydrosphingosine was extracted at room temperature with four 25-ml portions of chloroform-methanol 1/1 (v/v).

Identifiable derivatives of the free bases were obtained by acetylation with acetic anhydride in pyridine (21) or benzoylation according to the method of Carter *et al.* (24).

Triacetyl-erythro-sphingosine from phrenosin: m.p. 103°–105°; $[\alpha]_D^{32} = -9.4°$ (1.3% in chloroform).

Tribenzoyl-erythro-dihydrosphingosine from kerasin: m.p. 146°–148°; $[\alpha]_D^{32} = -30.20°$ (2.34% in chloroform).

Tribenzoyl-erythro-dihydrosphingosine from Gaucher cerebroside: m.p. 146°–148°; $[\alpha]_D^{32} = -29.95°$ (2.54% in chloroform).

In order to account for maximum amounts of the kerasin and Gaucher cerebroside bases, the mother liquors which remained from the preparation of their polybenzoyl derivatives were concentrated and hydrolyzed in methanolic KOH. The bases were then reisolated and acetylated.

Triacetyl-erythro-dihydrosphingosine from kerasin: m.p. 99°–101°.

TABLE 3. STEPWISE DEGRADATION OF CEREBROSIDES

Compound	Over-all Yield from Cerebroside		
	Phrenosin	Kerasin	Gaucher Cerebroside
	per cent	*per cent*	*per cent*
Oxidized cerebroside	95.4	99.6	99.9
$NaBH_4$ reduction product	86.0	97.1	96.5
Ceramide (crude)	78.0	96.8	96.5
Ceramide (purified)		78.2	74.3
Free base (crude)	78.0	96.8	95.3
Triacetyl-erythro-sphingosine	60.8		
Tribenzoyl-erythro-dihydrosphingosine		66.2	64.9
Tribenzoyl-erythro-dihydrosphingosine plus Triacetyl-erythro-dihydrosphingosine		88.6	81.4

Triacetyl-erythro-dihydrosphingosine from Gaucher cerebroside: m.p. 98°–100°.

The yields of these derivatives are summarized in Table 3.

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Studies on the biosynthesis of cholesterol: XIII. phosphomevalonic kinase from liver

HERMIONE HELLIG and G. POPJÁK

Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W. 12, England

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SUMMARY

The enzyme, phosphomevalonic kinase, which catalyzes the formation of 5-pyrophosphomevalonate from 5-phosphomevalonate and ATP has been purified from pig liver. The reaction is reversible, the position of equilibrium lying on the side of the forward reaction, and goes at optimum rate at pH 7.3 in the presence of 5 mM Mg^{++}, 3.6 mM ATP, and 1 mM 5-phosphomevalonate. The effect of various metal ions and inhibitors on the enzyme is described. The K_m for the enzyme is 0.3 mM. The preparation and some of the properties of 5-pyrophosphomevalonic acid are described.

Phosphomevalonic kinase catalyzes the reaction (−)5-phosphomevalonate + ATP ⇌ 5-pyrophosphomevalonate + ADP, the second biosynthetic step in the conversion of mevalonic acid into cholesterol. The enzyme has been partially purified from yeast (1, 2), and was also found in extracts of pig liver (3) during the purification of liver mevalonic kinase. This enzyme has now been purified further from pig liver and its properties studied in detail.

MATERIALS AND METHODS[1]

Biochemical Reagents. The sources of all biochemical reagents (ADP, ATP, ITP, DPNH, PEP, pyruvic kinase from muscle, lactic dehydrogenase, unlabeled DL-mevalonic acid lactone, and DL-mevalonic acid lactone-2-C^{14} and -1-C^{14}) were those given in another communication (3). N-ethylmaleimide, PCMB, and *o*-iodosobenzoate were supplied by L. Light and Co., Colnbrook, England; iodoacetamide was obtained from British Drug Houses, Ltd., Poole, Dorset.

Substrate. Optically active (−) 5-P-MVA-2-C^{14} and -1-C^{14} (4) were made with purified liver mevalonic kinase according to Levy and Popják (3); the preparations were free from ATP, ADP, and from mevalonate. The labeled 5-P-MVA preparations were made from C^{14}-mevalonate with specific activities of either 0.2, 0.1, or 0.002 μc per μmole. The specific activities of the 5-P-MVA preparations were, of course, the same as those of the mevalonates. The 5-P-MVA with the very low specific activity was used as substrate in the spectrophotometric assays of phosphomevalonic kinase, and the preparations with the higher specific activities in the "radiochromatographic" method.

Preparation of ATP^{32}. ATP, labeled with P^{32} terminally, was made by the oxidative phosphorylation of ADP with P^{32}-orthophosphate and rat liver mitochondria, which were prepared essentially according to the method of Schneider (5), and which showed strict respiratory control. Oxygen was bubbled through 30 ml of a 5 mM solution of K-phosphate buffer (pH 7.4, specific activity 13.3 μc P^{32} per μmole) containing 5 mM potassium glutamate; the osmolarity of the solution was adjusted to 0.26 with KCl. After 2 minutes, 2 ml of the mitochondrial suspension in isotonic sucrose was added to the mixture and then 9 μmoles of ADP were introduced. After 1 minute incubation at 25°, the reaction mixture was deproteinized with ice-cold perchloric acid (5% final concentration). It was ascertained in preliminary small-scale experiments that all the ADP became phosphorylated in 1 minute under the above conditions. The ATP^{32} was isolated from the deproteinized incubation mixture (after removal of perchloric acid as potassium perchlorate) by adsorption onto acid-washed charcoal (Nuchar C 190). Inorganic P^{32} was removed by extensive washing of the charcoal with water, and

[1] The following abbreviations are used: ADP and ATP, adenosine di- and triphosphate; ITP, inosine triphosphate; PEP, phosphoenolpyruvate; DPN and DPNH, diphosphopyridine nucleotide and its reduced form; BAL, 2,3-dimercaptopropanol; tris, tris(hydroxymethyl)aminomethane-HCl buffer; EDTA, ethylenediamine-tetraacetate; PCMB, *p*-chloromercuribenzoate; MVA, mevalonic acid; 5-P-MVA, 5-phosphomevalonic acid; 5-PP-MVA, 5-pyrophosphomevalonic acid.

the ATP^{32} eluted with 50% aqueous pyridine. The pyridine was extracted with ether and the preparation lyophilized; 6.5 μmoles of ATP^{32} were obtained. The specimen was free from ADP and was contaminated with only 0.5% of inorganic P^{32}. The ATP^{32} was diluted for use with unlabeled nucleotide to a specific activity of 0.2 μc per μmole.

Calcium Phosphate Gel. The calcium phosphate gel used for the adsorption of protein was made by the method of Tsuboi and Hudson (6). The suspensions contained 27 to 30 mg of dry matter per ml and were used 3 months after preparation.

Paper Chromatography. Two main systems were used for descending chromatography on Whatman No. 1 paper at 25° for about 16 hours. The solvent system A, isobutyric acid-aqueous NH_3 (sp gr 0.88)-water (66/3/30, v/v), gave the most reproducible R_f values. Solvent B, *tert*-butanol-formic acid-water (40/10/16, v/v) (2), was used mainly on a semimicro preparative scale to separate the adenine nucleotides from 5-PP-MVA. The $1^1/_2$-inch strips were scanned for radioactivity with an automatic recording device described previously (7). Chromatograms containing substances labeled with P^{32} and C^{14} were scanned twice, both with and without aluminum foil, 25 mg per cm^2, placed between the strip and the G-M counters. With the foil, C^{14} was not detectable and the efficiency of counting for the P^{32} was reduced by only 7% of that obtained without foil. Alternatively, autoradiograms were made after exposing Ilfex X-ray films to the chromatograms for 70 hours. Nucleotides were detected by photographic printing of the strips with ultraviolet light (8).

Measurement of C^{14} and P^{32}. C^{14} was measured with thin mica-window Geiger counters on samples of 2 cm^2 area and negligible thickness. The efficiency of counting was about 6%. P^{32} was counted in a M.6 liquid counter[2] of 9 ml capacity, with an efficiency of 10.9%. The P^{32} counts were corrected for the decay of the isotope.

Crude Enzyme Preparations. The initial extract from pig liver was prepared according to Hele and Finch (9) in the same way as for the preparation of mevalonic kinase (3). The crude extract, filtered through cheesecloth, was treated with protamine sulfate (1.5 mg per ml of extract), and the precipitate removed by centrifuging. The clear "protamine supernatant" served as starting material for the purification of the enzyme described under Results. All steps in the purification were carried out at 0° to 5°.

[2] The M.6 liquid counter was made by Twentieth Century Electronics, Ltd., King Henry's Drive, New Addington, Surrey, England.

Enzyme Assays. Two methods were used, either singly or together. In the radiochromatographic assay (3) the incubation mixture was deproteinized by heating it at 100° for 1 minute and chromatographing a sample in system A. The product, 5-PP-MVA, was identified by its R_f of 0.21–0.27 (de Waard and Popják [10]). This was clearly distinguishable from 5-P-MVA (R_f 0.36), mevalonic acid (R_f 0.66), and mevalonolactone (R_f 0.75). For quantitative work, the peaks recorded with the automatic scanner were copied onto typewriting paper and cut out and weighed.

The spectrophotometric assay of phosphomevalonic kinase measures in the presence of pyruvic kinase and lactic dehydrogenase the ADP generated during the reaction (2). The oxidation of DPNH at 37° was followed at 340 mμ in a Beckman DK-2 recording spectrophotometer using 3-ml cells with a 1-cm light path.

After determination of the optimum conditions for the action of the enzyme, the assay mixture was made to contain in a volume of 5.9 ml 0.1 M tris or K-phosphate buffer pH 7.35, NaF 10 mM, $MgCl_2$ 5 mM, 10 mM cysteine (neutralized with KOH), 0.6 mM PEP, 0.2 mM DPNH, 3.6 mM ATP (neutralized with KOH), 52 μg lactic dehydrogenase, 200 μg pyruvic kinase, and 2 mg bovine serum albumin. When very crude preparations were assayed, 30 mM nicotinamide was added to the reaction mixture. The phosphomevalonic kinase was added last. After incubation at 37° for 2 minutes, 2.9 ml of the reaction mixture was transferred to the test cuvette and the remainder to the control. One-tenth milliliter of substrate (containing 2.2 or 3.4 μmoles of 5-P-MVA) was then added to the test cuvette to initiate the reaction. The cruder enzyme fractions still contained the 5-PP-MVA anhydrodecarboxylase which also utilizes ATP in the formation of isopentenylpyrophosphate (2). This did not invalidate the assay, as only initial rates were used to determine enzyme activity. When preparations were assayed for their mevalonic kinase content, the spectrophotometric method of Levy and Popják (3) was used. In the stoichiometric and equilibrium studies, ADP was determined spectrophotometrically by adding aliquots of the incubation mixture to the test cuvette containing 2.9 ml of the pyruvic kinase-lactic dehydrogenase assay system (see above) and excess DPNH.

Standard Incubation Mixtures. Unless stated otherwise, all incubation mixtures in the experiments on the properties of the enzyme were as described above when the spectrophotometric assay was used. When the radiochromatographic technique was employed, the incubation mixture in a volume of 1 ml contained 100 mM tris pH 7.35, 10 mM NaF, 5 mM $MgCl_2$, 5

mM cysteine (neutralized with KOH), 3.6 mM ATP, and 1 mM 5-P-MVA (specific activity either 0.1 μc per μmole, i.e., 16,000 cpm per μmole, or 0.2 μc per μmole, i.e., 32,000 cpm per μmole). The reaction was started by the addition of phosphomevalonic kinase. These incubations were carried out at 37° for 1 hour, except when stated otherwise.

RESULTS

Purification of Phosphomevalonic Kinase. Table 1 shows the purification of phosphomevalonic kinase in a typical experiment. To 3,140 ml of protamine supernatant (see Methods), obtained from 1.6 kg of pig liver, there was added 720 g of solid $(NH_4)_2SO_4$ to give 30% saturation. After standing for 1 hour, the precipitate (fraction I) was removed by centrifuging at 2,000 × *g*. To 3,350 ml of the supernatant there was added 355 g. of $(NH_4)_2SO_4$ (48% saturation). The precipitate (fraction II) was collected by centrifuging, and served as the main source of phosphomevalonic kinase. By the addition of 306 g of $(NH_4)_2SO_4$ to the supernatant from fraction II, a further precipitate containing phosphomevalonic kinase was obtained, but the specific activity of the preparation was only one-half of that of fraction II, and was therefore discarded. The precipitates I and II were dissolved in 70 and 300 ml, respectively, of 20 mM K-phosphate buffer pH 7.6 containing 1 mM EDTA, and dialyzed for 4 hours against two changes of 10 mM K-phosphate buffer pH 7.6 containing 1 mM EDTA. Fraction I contained 390 units[3] of mevalonic kinase of relatively high specific activity (0.098) and virtually no phosphomevalonic kinase. Fraction II contained 750 units of mevalonic kinase and 305 units of phosphomevalonic kinase in a volume of 525 ml. This fraction also contained traces of 5-pyrophosphomevalonic decarboxylase. The solution of fraction II containing 18.9 g protein was then treated with 13.32 g calcium phosphate gel which adsorbed very nearly all the mevalonic kinase, leaving the second kinase in the supernatant (720 ml), which was acidified in 30-ml batches to pH 5.2 with 2 N acetic acid. The inactive precipitate was centrifuged off and the pH of the supernatant adjusted to 7.4 with 1 N KOH. This preparation had a specific activity of 0.078 and was completely free of mevalonic kinase (which is partly denatured at low pH), but contained traces of 5-PP-MVA decarboxylase. This latter enzyme could be completely removed by a second $(NH_4)_2SO_4$ fractionation, the kinase being recovered in the fraction precipitating between 30% and 45% saturation. This was done only for the preparations used for the equilibrium studies of the phosphomevalonic kinase reaction as it resulted in some loss of enzyme.

TABLE 1. PURIFICATION OF PHOSPHOMEVALONIC KINASE*

Fraction	Protein	Specific Activity	Total Enzyme Units
	g		
Protamine supernatant	78.5	0.0053	415
Fraction II	18.9	0.0158	305
Gel supernatant	4.17	0.0366	146
Gel supernatant after acid treatment	1.67	0.078	130

* Preparation from 1,600 g pig liver. The enzyme was assayed by the spectrophotometric method.

Decarboxylase Assay. The preparations were assayed for the 5-pyrophosphomevalonic decarboxylase by incubating in two Warburg flasks 1 ml of the standard incubation mixture, but using 5 mM ATP, plus 3 mM PEP, 35 μg pyruvic kinase, and 1.05 mg of phosphomevalonic kinase, No. 12-2. To one flask, 1 μmole of 5-P-MVA-2-C^{14}, and to the other, 1 μmole (32,000 cpm) of the 1-C^{14} substrate was added. The incubations were carried out for the usual time under N_2, then 2 N acetic acid was tipped in from the side bulb of the flask to liberate CO_2 which was trapped in 40% KOH and precipitated subsequently as $BaCO_3$ and counted. Both incubation mixtures were deproteinized and chromatographed. The $BaCO_3$ from the incubation with 5-P-MVA-1-C^{14} contained a total of 1,120 cpm. This amount of decarboxylase did not affect the determination of optimum conditions for the activity of phosphomevalonic kinase.

Enzyme Preparations. In the various experiments to be described, four preparations of phosphomevalonic kinase were used: No. 10-5, specific activity 0.041; Nos. 10-6, specific activity 0.069, and 9–6, specific activity 0.051, both free of decarboxylase; and No. 12-2, specific activity 0.078.

Properties of Phosphomevalonic Kinase. Although the spectrophotometric assay can be used with crude preparations, it was not entirely satisfactory with such preparations because a rereduction of DPN occurred. With the purified enzyme the initial rates were always linear, the assays were reproducible, and the rate of reaction was proportional to the protein

[3] One enzyme unit of mevalonic and phosphomevalonic kinase is defined as that amount of enzyme which phosphorylates 1 μmole of substrate per minute at 37°, and the specific activity of the enzyme as μmoles substrate phosphorylated per minute per milligram protein at 37°.

concentration up to about 1.7 mg per ml, which is in excess of the amounts commonly used in the assays (Fig. 1). The enzyme lost only 20% of its activity during storage of its solution (10 mg protein per ml) at −15° for 6 months.

Effect of pH. Figure 2 shows the dependence of reaction rate on pH in K-phosphate and tris-HCl buffers as determined by the spectrophotometric assay. The pH optimum lies between 7.2 and 7.3, the rate being reduced by half at pH's 5.8 and 7.8.

Coenzyme Requirements. Figure 3 shows the requirement for ATP. The highest rate of the reaction was observed with 3.6 mM ATP in the presence of 5 mM Mg^{++}; concentrations of ATP up to 9.3 mM were not inhibitory. ITP could not replace ATP as a phosphate donor, as determined by the radiochromatographic assay.

Metal Ion Requirements. The enzyme needs a divalent metal ion for activation. Figure 4 demonstrates the superiority of Mg^{++} over Mn^{++} as the activating ion, the optimum concentration being 5 mM. Higher concentrations were inhibitory. Mn^{++} could not effectively replace Mg^{++}, as at the optimum Mn^{++} concentration of 3 mM the rate was only about 20% of that obtained with 5 mM Mg^{++}. The effectiveness

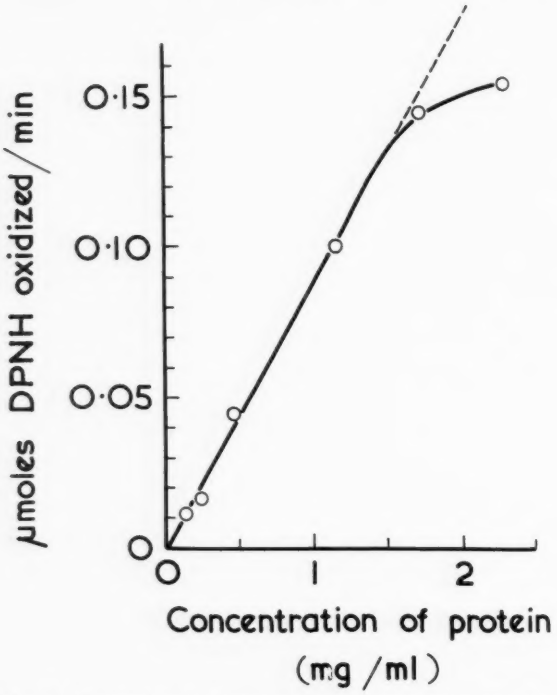


FIG. 1. Proportionality between protein concentration and rate of phosphorylation of 5-P-MVA as measured by the spectrophotometric assay. Concentration of ATP, 2 mM and of 5-P-MVA, 0.73 mM. Enzyme preparation No. 10-5.

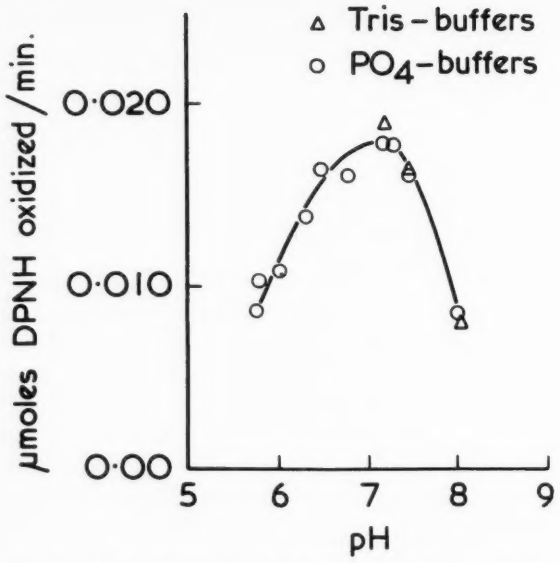


FIG. 2. Effect of pH on phosphomevalonic kinase activity. Standard spectrophotometric assays were used except that the concentration of ATP was 7.5 mM; and of 5-P-MVA, 0.73 mM. Enzyme preparation No. 12-2, 0.6 mg per assay.

of Mn^{++} was measured by the spectrophotometric assay in the presence of 0.13 mM Mg^{++} needed for the activation of the assay system. The "blank" value for this Mg^{++} concentration was subtracted from the final rates before plotting the graph. Cysteine was not used in the assay because it forms an insoluble complex with Mn^{++}.

The effects of various other metal ions on the phosphomevalonic kinase were tested in the presence of 5 mM Mg^{++} by the radiochromatographic assay. Ag^{+}, Cu^{++}, and Zn^{++} in 5 mM concentration completely inhibited the reaction, and Co^{++} was without effect.

Effect of Inhibitors. Standard 1-ml incubations were set up for the radiochromatographic assay without cysteine, using 1 mg of enzyme preparation 10-5 and 1 µmole of 5-P-MVA-2-C^{14}, specific activity 0.1 µc per µmole. The results are shown in Table 2. PCMB was the most potent inhibitor, its effect being partially reversed with 10 mM BAL or glutathione. Ten millimolar EDTA produced 61% inhibition, but KCN was without effect. It is of interest that although 5 mM N-ethylmaleimide and 5 mM *o*-iodosobenzoate caused 88% inhibition, 5 mM iodoacetamide gave only slight (9%) inhibition.

Effect of Substrate Concentration. The effect of changing the substrate concentration was determined by using the spectrophotometric assay. Figure 5 shows the increase in rate of reaction with increased concen-

trations of (−)5-P-MVA up to about 0.85 mM. The apparent Michaelis constant is approximately 0.3 mM.

Equilibrium and Reversibility of Reaction. The phosphomevalonic kinase reaction goes to completion only when the ADP formed during the reaction is rephosphorylated to ATP with phosphoenolpyruvate and pyruvic kinase. This was demonstrated both by spectrophotometric and radiochromatographic assays. When the ATP is not being regenerated, only a part of the 5-phosphomevalonate is phosphorylated to 5-pyrophosphomevalonate. In order to establish the equilibrium of the reaction, standard incubations for the radiochromatographic assay were set up with two phosphomevalonic kinase preparations, which were free from 5-pyrophosphomevalonic decarboxylase. The concentration of substrate (5-P-MVA-2-C^{14}) was 1 mM, and that of ATP was varied from 3 to 7.5 mM. The incubations were continued beyond the time needed for complete phosphorylation of the substrate when ATP was regenerated with PEP and pyruvic kinase; the reaction was therefore considered to have reached equilibrium. Table 3A and Figure 6 show the equilibrium to be well on the side of the forward reaction.

When in experiments, similar to those described above, 1 μmole of 5-PP-MVA-2-C^{14} (see preparation of 5-PP-MVA-2-C^{14}) and ADP were incubated with phosphomevalonic kinase without ATP, the reaction was reversed and 5-P-MVA and ATP were formed (Table 3B and Fig. 7). The reversibility of the phosphomevalonic kinase reaction was reported previously by both Lynen's and Bloch's groups working with the yeast enzyme (1, 2).

Stoichiometry of the Phosphomevalonic Kinase Reaction. A 3-ml standard incubation for radiochromatographic assay was set up containing 1 mM 5-P-MVA-2-C^{14}, 3 mM ATP, and 1 mg of enzyme preparation No. 10-5. At intervals aliquots of the incubation mixture were assayed for ADP spectrophotometrically and for extent of reaction by radiochromatography. As is shown in Table 3C, the amount of ADP produced was equal to the amount of 5-PP-MVA formed.

In another experiment 1 mM 5-P-MVA-2-C^{14} (specific activity 0.2 μc per μmole) was incubated with 5 mM P^{32}-ATP (specific activity 0.2 μc per μmole) and with 1 mg of preparation No. 9-6 in a 1-ml standard

TABLE 2. Effect of Various Substances on Phosphomevalonic Kinase*

Reagent	Concentration	Inhibition
	mM	*per cent*
EDTA	10	61
KCN	10	0
Iodoacetamide	5	9
N-ethylmaleimide	5	88
Na arsenite	5	0
BAL	10	0
Glutathione	10	0
Cysteine	10	0
PCMB	0.1	100
PCMB	1.0	100
PCMB + BAL	1.0 + 10.0	32
PCMB + glutathione	1.0 + 10.0	10
o-Iodosobenzoate	5	88

* Standard radiochromatographic assays were used with 1 mg enzyme No. 10-5 and 1 mM 5-P-MVA-2-C^{14}.

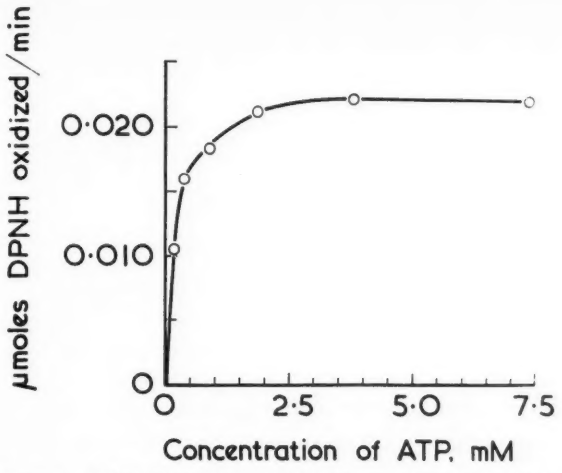


Fig. 3. Effect of ATP concentration on phosphomevalonic kinase reaction. Standard spectrophotometric assays were used with varying ATP concentration, 5-P-MVA, 0.73 mM; enzyme preparation No. 12-2, 0.6 mg per assay.

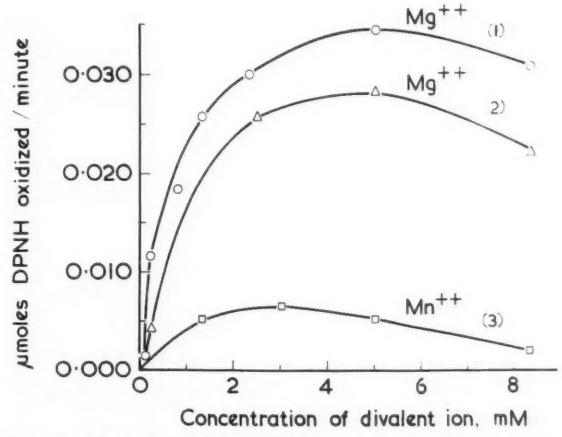


Fig. 4. Effect of Mg^{++} and Mn^{++} ions on phosphomevalonic kinase reaction. Standard spectrophotometric assays were used without cysteine, 5-P-MVA, 1.1 mM; enzyme preparation No. 12-2, 1.0 mg per assay. The data of curve (*1*) were obtained with the fresh preparation and those for curves (*2*) and (*3*) with the same preparation after 160 days of storage in solution at −15°.

TABLE 3. THE EQUILIBRIUM, REVERSIBILITY, AND STOICHIOMETRY OF THE PHOSPHOMEVALONIC KINASE REACTION

A. Equilibrium of Reaction*

Expt.	Time of Incubation	ATP Concentration	Conversion of 5-P-MVA to 5-PP-MVA
	minutes	*mM*	*per cent*
1	60	3.63	76
2	120	3.00	76
3	120	5.00	79
4	120	7.50	79

B. Reversibility of Reaction*

Expt.	Time of Incubation	ADP Concentration	Conversion of 5-PP-MVA to 5-P-MVA
	minutes	*mM*	*per cent*
1	60	5	29
2	120	4	39
3	120	7.5	44
4	120	0.0	0

C. Stoichiometry of Reaction*

Expt.	Time of Incubation	5-PP-MVA Formed	ADP Formed
	minutes	*μmoles*	*μmoles*
1	16.5	0.32	0.30
2	28.0	0.45	0.46

* Experimental conditions are described in the text.

incubation mixture. The products were then assayed by the radiochromatographic method after development of the chromatograms in solvent system B which separates 5-P-MVA, 5-PP-MVA, and the adenine nucleotides satisfactorily. The chromatograms were scanned for C^{14} and for P^{32}; the 5-PP-MVA formed contained P^{32}, the ratio of C^{14} to P^{32} in it being 0.93.

All these experiments fully establish the reaction catalyzed by phosphomevalonic kinase as:

$$\text{5-P-MVA} + \text{ATP} \rightleftharpoons \text{5-PP-MVA} + \text{ADP}.$$

TABLE 4. PROGRESS OF HYDROLYSIS OF 5-PP-MVA AND OF 5-P-MVA TO MVA BY 0.5 N HCl AT 100°

Time of Hydrolysis	5-PP-MVA	5-P-MVA	MVA
minutes	*μmoles*	*μmoles*	*μmoles*
0	0.90	0.10	0.0
4	0.60	0.40	0.0
8	0.30	0.70	0.0
23	0.00	0.00	1.0

Preparation of 5-Pyrophosphomevalonate. A 300-ml incubation was set up containing reactants in the following final concentrations: tris, pH 7.35, 0.1 M; cysteine (neutralized with KOH), 10 mM; NaF, 10 mM; $MgCl_2$, 5 mM; ATP, 3.6 mM; PEP, 1.5 mM; pyruvic kinase, 35 μg per ml; 5-P-MVA-2-C^{14} (specific activity 0.1 μc per μmole), 1 mM; and a total of 280 mg phosphomevalonic kinase preparation No. 10-5. After 1 hour incubation at 37° the reaction mixture was deproteinized by heating in a boiling water bath for 90 seconds. According to radiochromatographic assay, all the 5-P-MVA-2-C^{14} was used up and the products of the reaction were 5-PP-MVA-2-C^{14} and a trace of isopentenylpyrophosphate and of ADP (ATP was regenerated with PEP and pyruvic kinase). The main problem in the purification of 5-PP-MVA was therefore its separation from relatively large amounts of ATP. For this purpose the deproteinized incubation was lyophilized, and the residue taken up in a small volume of water; insoluble material was removed by centrifugation. The clear solution was cooled in ice and its pH adjusted to 6.0. Then ethanol was added slowly to a concentration of 75%. The precipitate formed (mostly ATP and salts) was washed repeatedly with 75% ethanol, and the washings were combined with the main ethanol supernatant. It is important to adjust the pH of the solution to 6.0 before the ethanol precipitation, and also not to use concentrations of ethanol higher than 75%, otherwise a substantial proportion of the 5-PP-MVA may be precipitated with the ATP and salts. After removal of ethanol on a rotary evaporator at room temperature the aqueous solution was made alkaline (pH 8.5) with KOH, and 550 μmoles of $BaCl_2$ was added, which removed further amounts of ATP and only a little of 5-PP-MVA. After separation of the Ba salt of ATP by centrifugation, a further 500 μmoles of $BaCl_2$ and ethanol to a concentration of 50% were added to the solution in order to precipitate 5-PP-MVA as the Ba salt, which was collected by centrifugation and washed with 50% ethanol and absolute ethanol. The Ba salt of 5-PP-MVA was then dissolved in a minimum amount of dilute HCl and the Ba precipitated by the addition of a Na_2SO_4 solution. After removal of the $BaSO_4$ precipitate, the pH of the solution was adjusted to 6.9 with KOH. Assay of the preparation showed it to contain 170 μmoles of 5-PP-MVA-2-C^{14} free of any other radioactive substance, but contaminated with 85 μmoles of ATP. The residual ATP was removed by differential adsorption onto charcoal. The solution of the 5-PP-MVA was diluted to a concentration of 1.38 mM. Acid-washed charcoal (Nuchar C 190), 70 mg for each

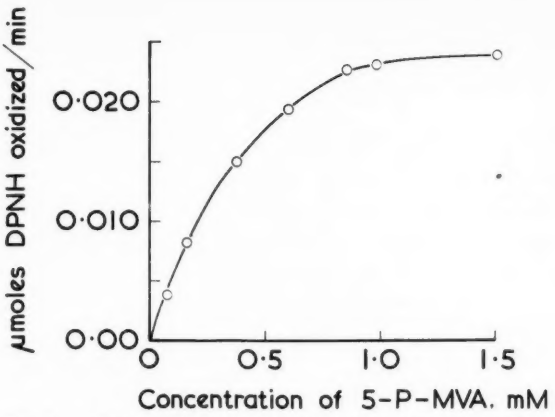


FIG. 5. Effect of substrate concentration on phosphomevalonic kinase reaction. Standard spectrophotometric assays were used with preparation No. 12-2, 0.6 mg per assay.

10 ml, was then stirred into the solution and removed by filtration after about 30 minutes. Preliminary small-scale experiments showed that the ATP was adsorbed first onto the charcoal, and that the small amounts of 5-PP-MVA that were also adsorbed could be eluted with water. After this simple treatment most of the ATP was removed, and only about 10% of the 5-PP-MVA was lost. For purposes of special experiments, e.g., for the study of the reversibility of the phosphomevalonic kinase reaction, when 5-PP-MVA completely free of ATP was needed, samples of the preparation were chromatographed on Whatman No. 1 paper in solvent system B in which 5-PP-MVA and ATP have R_f values of 0.33 and 0.10, respectively. The 5-PP-MVA was eluted from the paper with dilute aqueous ammonia. 5-PP-MVA, free from ATP, could also be prepared by the use of larger amounts of charcoal during the differential adsorption procedure or by repeating the Ba-precipitation; both these techniques, however, caused 20% to 30% losses of 5-PP-MVA.

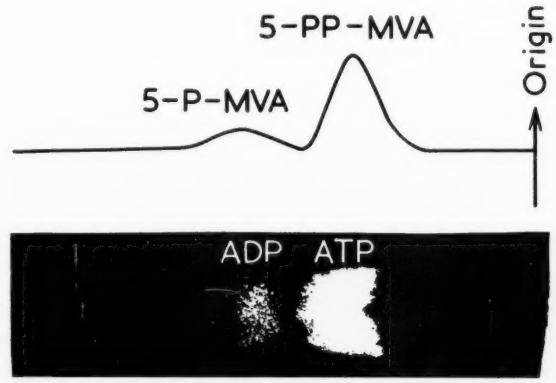


FIG. 6. Formation of ADP from ATP during phosphorylation of 5-P-MVA to 5-PP-MVA. The ATP used in the incubation contained no ADP as determined spectrophotometrically with the pyruvic kinase-lactic dehydrogenase assay system for ADP.

Characterization of 5-Pyrophosphomevalonate. This compound is identical with "Compound II" of Chaykin *et al.* (11) and with that described by de Waard and Popják (10) as diphosphomevalonate, and characterized by Bloch *et al.* (2) and Henning *et al.* (1) as the 5-pyrophosphomevalonate. The behavior of this substance, both on paper chromatograms and on ion

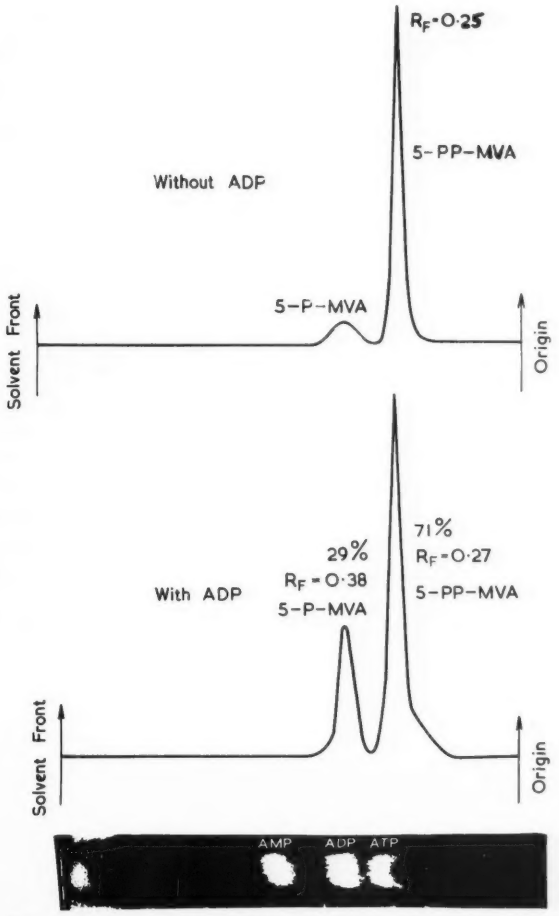


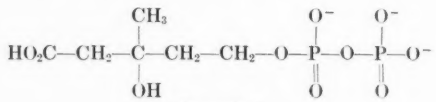
FIG. 7. Radiochromatographic evidence on the reversibility of phosphomevalonic kinase reaction. Standard 1-ml incubations were made with 1 μmole of 5-PP-MVA-2-C^{14} without and with 5 μmoles of ADP. After 1 hour incubation aliquots of the deproteinized incubations were chromatographed in solvent system A, and the paper strips scanned for radioactivity and printed with ultraviolet light. The lower graph and the ultraviolet print from the chromatogram shows that both 5-P-MVA and ATP were formed from 5-PP-MVA and ADP. The ADP used was free of ATP, but was contaminated with AMP (Expt. 1 of Table 3B).

exchange columns, has been described in detail (10). 5-PP-MVA is resistant to hydrolysis by 1 N KOH at 100° for 5 minutes, but it is hydrolyzed in 0.5 N HCl at 100° to MVA with 5-P-MVA as intermediate (Table 4). The mevalonic acid formed from the hydrolysis of 1 μmole of 5-PP-MVA-2-C^{14} (specific activity 16,000 cpm per 1 μmole) was mixed with 63.5 mg of unlabeled mevalonic acid and the benzhydrylamide prepared (12) from the mixture. After recrystallization, the benzhydrylamide (m.p. 96°) had a specific activity of 130 cpm per μmole.

5-Pyrophosphomevalonate as Precursor of Allyl Pyrophosphates. It has already been shown that 5-PP-MVA-2-C^{14} is converted into cholesterol more efficiently than DL-MVA-2-C^{14}, but that ATP was still needed in this conversion (10). Supplementing this evidence is our finding that 5-PP-MVA-2-C^{14} is readily converted into C^{14}-allyl pyrophosphates (mainly the pyrophosphates of geraniol and of farnesol), which were shown to be the precursors of squalene in liver (13) as well as in yeast enzyme preparations (14). A 1-ml incubation mixture was set up containing 1 μmole of 5-PP-MVA-2-C^{14} (specific activity 32,000 cpm per μmole), 100 μmoles of tris buffer, pH 7.4, 10 μmoles of NaF, 5 μmoles of $MgCl_2$, 5 μmoles of ATP, and 14 mg of a soluble enzyme fraction (F_{30}^{60}-enzymes) made from rat liver and which was shown to convert DL-MVA-2-C^{14} or (−)5-P-MVA-2-C^{14} into the allyl pyrophosphates. The mixture was incubated at 37° for 1 hour, at the end of which it was analyzed for allyl pyrophosphates according to the procedure of Goodman and Popják (13). One-half of the substrate (16,060 cpm) was converted into these substances, most of the radioactivity (85%) being associated with farnesyl pyrophosphate and a little with geranyl pyrophosphate.

DISCUSSION

Phosphomevalonic kinase is closely associated in liver extracts with mevalonic kinase; its separation from the latter enzyme is rendered the more difficult because usually one-quarter to one-half as much phosphomevalonic kinase as mevalonic kinase is found in the extracts. These two kinases differ from one another in that only ATP can serve as coenzyme for phosphomevalonic kinase, whereas liver mevalonic kinase functions with ATP and ITP almost equally well (3), and also in that Ca^{++}, which can activate mevalonic kinase, cannot activate the phosphokinase. The affinity of the phosphomevalonic kinase for its substrate (5-phosphomevalonate) is appreciably lower ($K_m = 3 \times 10^{-4}$M) than the affinity of mevalonic kinase for mevalonate ($K_m = 5 \times 10^{-5}$M). This circumstance suggests that the phosphokinase may be rate limiting in the conversion of mevalonate into squalene, although the favored equilibrium of the reaction may partially counteract this. As the phosphomevalonic kinase reaction is reversible, it is evident that the second phosphate bond in 5-PP-MVA is an energy-rich bond, probably of the same order of magnitude as that of ATP. The values for the free energy changes in the phosphomevalonic kinase reaction could not be calculated from our equilibrium data, as the "activities" of the phosphorylated derivatives of mevalonate in solutions of high ionic strength are unknown. All our data on 5-PP-MVA (the transfer of the terminal phosphate group of ATP to 5-P-MVA, reversibility of the phosphomevalonic kinase reaction, the formation of 5-P-MVA and of MVA from 5-PP-MVA by acid hydrolysis) are entirely consistent with earlier conclusions (1, 2) that in "diphosphomevalonate" (10) the second phosphate group is in an acid anhydride linkage with the phosphate in 5-P-MVA and that the product of the phosphomevalonic kinase reaction is the 5-diphospho(pyrophospho)-mevalonate (Formula I).



Formula I

The properties of liver phosphomevalonic kinase differ from the similar enzyme studied in yeast (1, 2). Whereas the liver enzyme showed a distinct pH optimum around 7.3, it was reported (2) for the yeast enzyme that its activity did not vary in the pH range of 5.5 to 10. Also, yeast phosphomevalonic kinase was activated by Zn^{++} almost as well as by Mg^{++}, whereas for liver mevalonic kinase, Zn^{++} ions were severely inhibitory.

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Studies on the biosynthesis of cholesterol: XIV. the origin of prenoic acids from allyl pyrophosphates in liver enzyme systems

J. CHRISTOPHE* and G. POPJÁK

Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W. 12, England

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SUMMARY

Allyl pyrophosphates (3,3-dimethylallyl, geranyl, and farnesyl pyrophosphate), which are known intermediates in the biosynthesis of squalene from mevalonate, are also metabolized in the liver by an alternative pathway to acids. The first step in the conversion of the allyl pyrophosphates into the acids (dimethylacrylic, geranoic, and farnesoic acids) is their irreversible dephosphorylation into free prenols by a microsomal phosphatase, which has its optimum pH at around 7.0. In a second step the free prenols are irreversibly dehydrogenated to the acids by liver alcohol dehydrogenase and aldehyde dehydrogenase present in a soluble protein fraction of liver homogenates. This dehydrogenation proceeds best at pH 7.5 and is inhibited by sulfhydryl reagents. The prenols are more specific substrates for liver alcohol dehydrogenase than is ethanol, but they cannot act as substrates for yeast alcohol dehydrogenase. The formation of allyl pyrophosphates, of free prenols, and of prenoic acids from mevalonate was also observed *in vivo*.

The biosynthesis of farnesoic acid[1] from mevalonate-2-C^{14} in liver enzyme preparations, synthesizing squalene and cholesterol, was first reported by Dituri *et al.* (1), and was confirmed in this laboratory (2, 3). Gas-liquid radiochromatographic analysis (4) of acids isolated from enzyme incubations revealed, however, that *trans-trans*-farnesoic acid was only one of several acids formed from mevalonate-2-C^{14} (3). Our interest in the biological synthesis of farnesoic acid from mevalonate was concerned with two possibilities, viz. (*a*) that this substance might be a precursor of squalene, as was suggested by Dituri *et al.* (1), or (*b*) that it originated by an alternative pathway of the metabolism of intermediates of squalene biosynthesis. The incorporation of C^{14} from biosynthetically prepared C^{14}-farnesoic acid into cholesterol, reported by Dituri *et al.* (1), could not be confirmed in our laboratory (3), and farnesoic acid was therefore excluded as an intermediate in squalene biosynthesis. Since the pyrophosphates of dimethylallyl alcohol, of geraniol, and of farnesol ("allyl pyrophosphates") had been identified as intermediates in the synthesis of squalene, both in yeast and in liver enzyme systems (5 to 9), it seemed most probable that the acids synthesized from mevalonate in liver enzyme preparations were in fact products of an alternative metabolism of these intermediates, as has already been suggested (3).

It will be shown in this paper that dimethylacrylic, geranoic, and farnesoic acids are formed in liver enzyme preparations from allyl pyrophosphates by a combined action of a phosphatase, present mainly in the microsomes, and of two dehydrogenases.

METHODS AND MATERIALS

Enzyme Preparations. Soluble supernatants and microsomes were prepared as described previously (10, 11) from rat liver homogenates made according to Bucher and McGarrahan (12). A fraction of the soluble supernatant, F_{30}^{60}, was a preparation similar to that described previously, and shown to synthesize the pyrophosphates of dimethylallyl alcohol, of geraniol, and of farnesol from DL-mevalonate-2-C^{14}, or from (−)-5-phosphomevalonate-2-C^{14}, and ATP in the presence of Mg^{++} (9). In a few experiments, the proteins of the soluble supernatant of rat liver homogenates were precipitated with $(NH_4)_2SO_4$ (90% saturation), and

* Holder of a Wellcome Trust Fellowship and in receipt of a grant from the Institut Interuniversitaire des Sciences Nucléaires, Belgium.

[1] This substance has previously been referred to as farnesenic acid. The present term is preferred since the acid should be considered as derived from farnesol.

this unfractionated protein preparation (F_0^{90}) was used —after being dissolved in 0.02 M $KHCO_3$ and dialyzed (11)—as a source of enzymes. In addition to the F_{30}^{60}-enzymes, fractions of the soluble supernatant of homogenates precipitable between 0% and 30%, and between 60% and 90% saturation with $(NH_4)_2SO_4$ (F_0^{30}- and F_{60}^{90}-preparations, respectively) were also tested.

Suspensions of microsomes were stored at −15°, and retained their activity for periods of at least 3 months. In one experiment, microsomes were extracted with Lubrol-W, a nonionic detergent (Imperial Chemical Industries Ltd., England), and a Lubrol-insoluble and a Lubrol-soluble fraction was obtained. The usual suspension of microsomes (11) was diluted with an equal volume of 0.1 M potassium hydrogen phosphate buffer, pH 7.4, and a 10% aqueous solution of Lubrol-W was added to it in small portions up to a final concentration of 1%. The suspension, kept at 0°, was homogenized repeatedly during 30 minutes in a loosely fitting homogenizer; it was then centrifuged at 105,000 × *g* for 1 hour. The sedimented pellet was resuspended in the 0.1 M phosphate buffer containing 1 mM EDTA[2]; the resuspended particles and the Lubrol-soluble supernatant were dialyzed for 3 hours against the same medium. Approximately one-half of the microsomal proteins was rendered soluble by the Lubrol treatment.

DL-*Mevalonic Acid Lactone-2-C*^{14}. This was purchased from the Radiochemical Centre, Amersham, Bucks, England, and was converted to the potassium salt by hydrolysis with a small excess of KOH at 37°. The optically active (−)5-phosphomevalonate-2-C^{14} was prepared with purified liver mevalonic kinase according to Levy and Popják (13).

C^{14}*-Allyl Pyrophosphates.* These were synthesized enzymically from (−)5-phosphomevalonate-2-C^{14} (26,000 cpm/μmole) and purified according to Goodman and Popják (9). After complete hydrolysis of a sample of the allyl pyrophosphates by calf intestinal phosphatase at pH 8.6, the alcohols liberated were extracted with petroleum ether. Gas-liquid radiochromatographic analysis of these alcohols showed that they consisted of 3% dimethylallyl alcohol, 8% geraniol, 11% nerolidol, 74% *trans-trans*-farnesol, and of some substances with retention volumes greater than that of 4% farnesol. Thus the preparation consisted mostly of C^{14}-farnesyl pyrophosphate.

C^{14}*-Prenols.*[3] These were prepared in three different ways from C^{14}-allyl pyrophosphates. In the first procedure, the purified preparation of the allyl pyrophosphates was hydrolyzed with prostatic acid phosphatase (9) and the liberated alcohols (mostly farnesol, see above) extracted with petroleum ether.

In the second procedure, C^{14}-allyl pyrophosphates were first synthesized from DL-mevalonate-2-C^{14} with the F_{30}^{60}-enzymes (9) and then, without isolation, were hydrolyzed by the addition of a suspension of microsomes (0.05 ml/ml of incubation) and of 1 mM PCMB; after 90 minutes of incubation with the microsomes at 37°, the C^{14}-prenols were extracted with petroleum ether. The use of PCMB prevented the conversion of the prenols to acids by the dehydrogenases present in the F_{30}^{60}-preparations (see also under Results and Discussion). Analysis of the C^{14}-prenols by gas-liquid radiochromatography showed them to consist of 2% dimethylallyl alcohol, 12% geraniol, and of 73% *trans-trans*-farnesol, together with a little nerolidol and some other unidentified substances.

In a third procedure, C^{14}-allyl pyrophosphates were hydrolyzed for 16 hours by acid (0.2 N HCl) at room temperature, and the prenols extracted with petroleum ether. It was shown previously (9) that hydrolysis of the allyl pyrophosphates by acid causes an allylic rearrangement, most of the farnesyl pyrophosphate being converted into nerolidol. In the particular preparation, 65% of the radioactivity was associated with nerolidol, 20% with farnesol, and the remainder with C_5, C_{10}, and with some other alcohols.

The C^{14}-prenols were kept (without addition of carrier) in petroleum ether at −15°; when needed as substrates in enzyme incubations, samples of their solution were slowly evaporated under a stream of N_2 in the incubation vessel.

Incubations. Most of the incubations (1 ml) were done at 37° in 10-ml glass-stoppered centrifuge tubes. For "anaerobic" incubations, the tubes were flushed with N_2 and immediately stoppered.

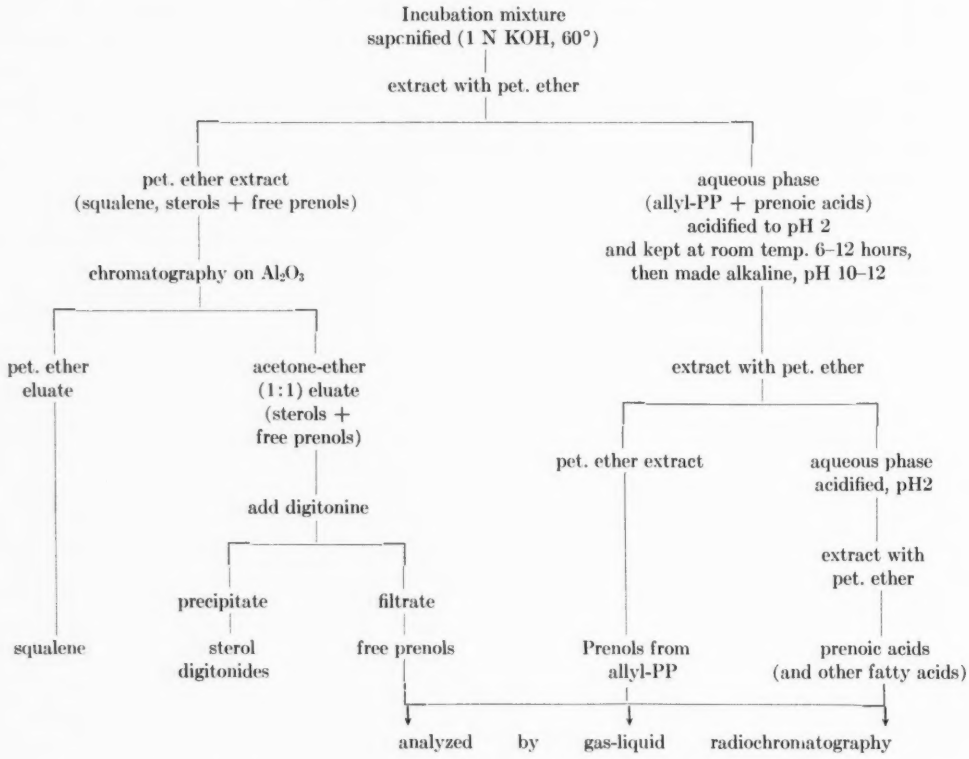
Isolation and Analysis of Incubation Products. The

[2] The following abbreviations are used: ATP, ADP, adenosine-5′-tri- and diphosphate; DPN, TPN, DPNH, TPNH, di-, triphosphopyridine nucleotide, oxidized and reduced; EDTA, ethylenediamine-tetraacetate; tris, tris(hydroxymethyl)aminomethane; PEP, phosphoenolpyruvate; GSH, reduced glutathione; PCMB, *p*-chloromercuribenzoate; BAL, 2,3-dimercaptopropanol; allyl-PP, allyl pyrophosphates.

[3] The terms "prenol" and "prenoic acid" have been proposed by Popják and Cornforth (14) to denote alcohols and acids, respectively, which can be clearly dissected into isoprenoid units, $—CH_2—C(CH_3)=CH—CH_2—$. According to this nomenclature, which may be particularly useful in view of the increasing number of recognized long-chain polyisoprenoid substances, 3,3-dimethylallyl alcohol is denoted as monoprenol, geraniol as *trans*-diprenol, farnesol as *trans-trans*-triprenol, farnesoic acid as triprenoic acid, etc. The tertiary alcohols, linalool and nerolidol (isomers of geraniol and of farnesol) may be called *tert*-diprenol and *tert*-triprenol, respectively. Collectively they may be referred to as prenoid substances.

incubation mixtures were hydrolyzed at 60° for 1 hour after addition of 1 ml of 2 N KOH in 70% ethanol. The hydrolysis and the extraction of all enzymic products were carried out in the centrifuge tubes. In various experiments the following products were determined: squalene, cholesterol, allyl pyrophosphates, free prenols, and prenoic acids. All these products can be determined in one incubation. The methods used were essentially those described previously (9); the outline of the whole procedure is given in Chart 1.

CHART 1. Flowsheet of Procedures Used for Isolation of Enzymic Products Biosynthesized from Mevalonate-2-C^{14}



For purposes of gas-liquid chromatography, the acids were methylated with diazomethane. The analysis and identification of prenols and of prenoic acids by gas-liquid chromatography has been described (4, 14), and was made with the gas-density balance (15) or with the Argon detector (W. G. Pye & Co., Cambridge, England). Since the amounts of the biosynthetic C^{14}-prenols and C^{14}-prenoic acids were too small for detection by the gas-density balance, gas-liquid radiochromatography (4, 9) was the method employed, and a mixture of unlabeled prenols (dimethylallyl alcohol, linalool, geraniol, nerolidol, *cis-trans*-farnesol, and *trans-trans*-farnesol), in the ratios of 1/1/1/2/2/2 (v/v) or, as the occasion required, varying amounts of the methyl esters of unlabeled prenoic acids (3,3-dimethylacrylic, *cis*-geranoic, *trans*-geranoic, *cis-trans*-farnesoic, and *trans-trans*-farnesoic acids) were used as internal standards.

Spectrophotometric Studies. The dehydrogenation of prenols and of other alcohols by crystalline alcohol dehydrogenase from liver and yeast, and by dehydrogenases present in the F_{30}^{60}-enzyme preparations was measured by following the reduction of DPN at 340 mμ and at 37° in a recording Beckman-DK2 spectrophotometer equipped with a time drive and an electrically heated cell carrier. The reactions were started by the addition of the alcohol, the change in light extinction being recorded against a reference cuvette containing all ingredients except the alcohol.

In one set of experiments, substrate specificity for liver and yeast alcohol dehydrogenase was determined by measuring the initial rates of DPN reduction in the presence of various alcohols. Each cuvette contained 10 μmoles of of alcohol, 0.6 μmole of DPN, and 15 μg of

alcohol dehydrogenase in a final volume of 3 ml. In a second set, the equilibrium of DPN reduction was studied. Here each cuvette contained DPN (1.5 μ moles) in excess of the substrate alcohols (0.05–0.15 μmole), and 75 μg of alcohol dehydrogenase in a final volume of 3 ml.

The suspensions of crystalline alcohol dehydrogenases (from liver or from yeast) were dissolved on the experimental day in 2% bovine serum albumin. Among the various alcohols used, farnesol and geraniol have low solubility in water and therefore these alcohols were suspended in 0.1 M concentration in 2% bovine serum albumin solution; all other alcohols were added to the reaction mixture from a 0.1 M aqueous solution.

Sources of Enzymes, Coenzymes, and Other Substances. Crystalline horse liver alcohol dehydrogenase and crystalline yeast alcohol dehydrogenase were obtained from Boehringer & Söhne, Mannheim, Germany. The sources of all other biochemical reagents, enzymes, coenzymes, and of various chemicals were given in previous communications (9, 13). The origin and purity of specimens of prenols and of the methyl esters of prenoic acids has also been described (14). Dr. A. Eschenmoser of the Eidgenössische Technische Hochschule, Zurich, provided specimens of cyclized geranoic and cyclized farnesoic acid.

RESULTS

It has already been recorded (7, 9) that incubation of F_{30}^{60}-enzymes with DL-mevalonate-2-C^{14}, ATP, and Mg^{++} leads principally to the synthesis of allyl pyrophosphates with the simultaneous appearance of only small amounts (5% to 15% of the allyl pyrophosphates formed) of acidic substances. When, however, microsomes are added to an F_{30}^{60}-enzyme incubation in which the allyl pyrophosphates have already been synthesized during a preliminary incubation, a substantial part of the allylic derivatives is converted into carboxylic acids (Table 1). The Table also shows the appearance of C^{14} in free prenols, mainly in *trans-trans*-farnesol according to gas-liquid radiochromatographic analysis. This finding suggested that the radioactive acids were derived from the allyl pyrophosphates in two steps: dephosphorylation of the allyl pyrophosphates to free prenols, followed by dehydrogenation of the alcohols to acids. This assumption was borne out by the results of the experiment recorded in Table 2, in which a purified specimen of C^{14}-allyl pyrophosphates (9) was incubated with microsomes alone and with microsomes plus F_{30}^{60}-enzymes. The microsomes caused an almost complete hydrolysis of the allylic derivatives into free prenols; significant amounts of C^{14}-acids appeared only when F_{30}^{60}-enzymes were also added to the incubations. It was found, further, that the enzymes responsible for the formation of C^{14}-acids were almost entirely confined to a protein fraction of the soluble supernatant of rat liver homogenates precipitable with $(NH_4)_2SO_4$ between 30% and 60% saturation (Table 3).

These observations indicated, therefore, that the following sequence of reactions could account for the synthesis of C^{14}-acids from mevalonate-2-C^{14} in liver enzyme preparations: synthesis of allyl pyrophosphates, their subsequent hydrolysis by a microsomal phosphatase, and, finally, the dehydrogenation of the free prenols to acids by soluble enzymes. In the remainder of this paper detailed studies of the last two reactions are presented.

TABLE 1. EFFECT OF RAT LIVER MICROSOMES ON SYNTHESIS OF PRENOIC ACIDS FROM MEVALONATE-2-C^{14}*

Incubation	C^{14} Found in		
	Allyl-PP	Prenoic Acids†	Free Prenols
	cpm	*cpm*	*cpm*
Without microsomes	49,700	1,400	90
Microsomes added after 1 hour	17,600	36,400	3,615

* Each tube contained in a final volume of 5 ml, 1.0 mM DL-mevalonate-2-C^{14} (total of 177,000 cpm, i.e., 88,500 counts of the biologically active [+] mevalonate), 5 mM $MgCl_2$; 7.5 mM ATP; 10 mM NaF; 0.1 M phosphate buffer, pH 7.4, and F_{30}^{60}-enzymes (21 mg protein/ml). Incubations under N_2 for 2 hours at 37°. After 1 hour, microsomes (0.20 ml) were added to one of the tubes.

† Although this acidic fraction as isolated from the incubations contained also the various normal fatty acids, none of the radioactivity was associated with these, but only with prenoic acids. In every experiment the prenols as well as the acids were analyzed by gas-liquid radiochromatography.

TABLE 2. HYDROLYSIS OF C^{14}-ALLYL PYROPHOSPHATES BY RAT LIVER MICROSOMES AND EFFECT OF SOLUBLE ENZYMES ON PRENOLS*

Addition	C^{14} Found in		
	Residual Allyl PP	Free Prenols	Prenoic Acids
	cpm	*cpm*	*cpm*
None	340	5020	440
F_{30}^{60}-enzymes†	220	2900	2260

* Each tube contained in 1 ml, 5,500 cpm of C^{14}-allyl pyrophosphates (4,100 cpm, i.e., 0.053 μmole of farnesyl pyrophosphate), 0.05 ml microsomes, 5 mM $MgCl_2$, and 0.1 M tris-HCl buffer, pH 7.4. Incubation in air for 1 hour at 37°.

† 15 mg protein.

TABLE 3. PRODUCTION OF PRENOIC ACIDS FROM C^{14}-ALLYL PYROPHOSPHATES BY MICROSOMES AND SOLUBLE ENZYME FRACTIONS*

Soluble Enzymes Added		C^{14} Found in		
Fraction	Protein	Residual Allyl-PP	Free Prenols	Prenoic Acids
	mg	*cpm*	*cpm*	*cpm*
F_0^{90}	13	520	1200	1500
F_0^{30}	13	300	3040	320
F_{30}^{60}	13	920	1000	1380
F_{60}^{90}	6.4	820	1880	360

* Each tube contained in 1 ml, 3,750 cpm of C^{14}-allyl pyrophosphates (2,800 cpm, i.e., 0.036 μmole of farnesyl pyrophosphate); 0.05 ml microsomes; 5 mM $MgCl_2$; 0.1 M phosphate buffer, pH 7.4; and fractions of a 105,000 × *g* supernatant of liver homogenate precipitated with $(NH_4)_2SO_4$ between various percentages of saturation. Incubation under N_2 for 1 hour at 37°.

Properties of the Microsomal Allyl Pyrophosphatase. Phosphate buffer was avoided in experiments designed to test the properties of the microsomal phosphatase since high concentrations (0.1 M) of phosphate were found to inhibit the enzyme.

For the determination of the pH optimum of this phosphatase, Na-citrate-HCl buffers (0.1 M, pH range 5.6 to 8.1) and tris-HCl buffers (0.1 M, range 6.9 to 9.0) were used. The optimum pH was around 7.0 (Fig. 1). The activity of the enzyme decreased fairly rapidly on the alkaline side, so that at pH 9.0 only 42% of the maximum activity could be demonstrated; on the acid side, however, there was some indication of a second peak of activity developing below pH 5.6. As the allyl pyrophosphates are unstable below pH 5.5 and cleave into inorganic pyrophosphate and prenols (9), measurements of enzymic hydrolysis at more acid pH values would be meaningless. The extent of the enzymic hydrolysis of the allyl pyrophosphates was measured by the amount of free prenols appearing, and not by release of phosphate; we have no data to show whether inorganic orthophosphate or pyrophosphate is liberated by the microsomal phosphatase.

Figure 2 illustrates the rate of hydrolysis of the allyl pyrophosphates by microsomes at pH 7.4; 50% of the substrate was hydrolyzed in 18 minutes, and 80% in 1 hour.

The microsomal allyl pyrophosphatase needed no Mg^{++} or other divalent metal ion for activation. The activity of the microsomes was not altered by dialysis for 5 hours against 0.02 M tris-HCl buffer, pH 7.4, containing 1 mM EDTA. Even when such dialyzed preparations were pretreated at 0° for 70 minutes with 5 mM EDTA, KCN, 8-hydroxyquinoline-5-sulfonic acid, or with 1,10-phenanthroline, the phosphatase activity was still the same as that of the untreated microsomes. In fact, with the exception of Mg^{++}, all the divalent ions tested (Ca^{++}, Ni^{++}, Co^{++}, Mn^{++}, Cu^{++}, and Zn^{++}, in order of increasing effectiveness) were inhibitory in a concentration of 5 mM (Table 4). A number of other substances, listed in Table 4, were also inhibitory, the most potent ones being NaF and, most surprisingly, crystalline bovine serum albumin. The F_{30}^{60}-enzymes containing 15 mg protein per ml, 2 mM glycine plus 10 mM glucose-6-phosphate, 0.1 M sodium pyrophosphate, 10 mM ATP, 2 mM dimethylallyl alcohol, 1 mM PCMB, and 1 mM N-ethyl maleimide had no effect on the hydrolysis of C^{14}-allyl pyrophosphates.

The extraction of microsomes with Lubrol-W (see under Methods) showed that the allyl pyrophosphatase was firmly attached to these particles. Although the treatment with the detergent dissolved about one-half of the microsomal proteins, nevertheless almost all the phosphatase activity remained in the Lubrol-insoluble particles.

When C^{14}-prenols (mostly C^{14}-farnesol, see under Methods) were incubated either with microsomes, or with microsomes plus F_0^{90}-enzymes, under conditions in which farnesyl pyrophosphate and mevalonate,

TABLE 4. INHIBITORS OF MICROSOMAL PHOSPHATASE*

Additions	Inhibition†
	per cent
$MgCl_2$, 5 mM	0
$CaCl_2$, 5 mM	14
$NiSO_4$, 5 mM	27
$CoCl_2$, 5 mM	56
$MnCl_2$, 5 mM	71
$Cu(NO_3)_2$, 5 mM	90
$ZnSO_4$, 5 mM	93
NaF, 5 and 10 mM	59
Potassium phosphate‡	17
Sodium arsenate‡	17
BSA,§ 0.4 mg/ml	54
BSA,§ 2.0 mg/ml	78
BSA,§ 4.0 mg/ml	81

* Each incubation contained in 1 ml, 2,500 cpm of C^{14}-allyl pyrophosphates (1,900 cpm, i.e., 0.024 μmole, in farnesyl pyrophosphate), 0.1 M tris-HCl buffer, pH 7.4, and 0.05 ml microsome suspension dialyzed against 1 mM EDTA in 0.02 M tris-HCl buffer, pH 7.4, for 5 hours at 5°. Additions and their concentrations were as shown. Aerobic incubations for 1 hour at 37°.

† The per cent inhibitions were calculated from a comparison of the hydrolysis of allyl pyrophosphates found in the absence of additions.

‡ pH 7.4, 0.1 M.

§ Bovine serum albumin.

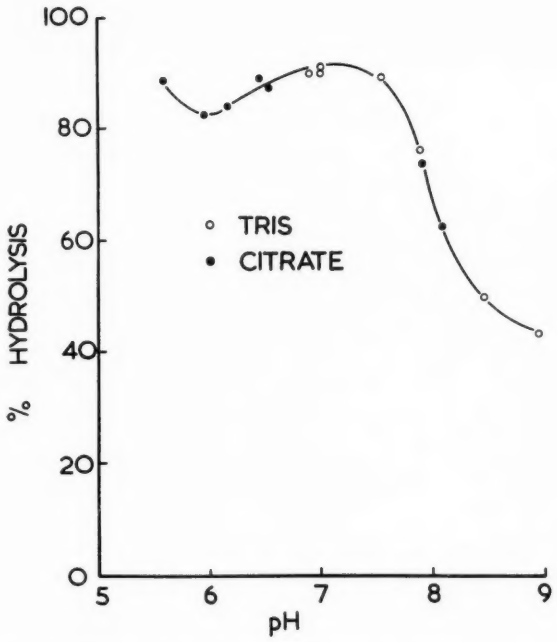


FIG. 1. Relation between pH and hydrolysis of allyl pyrophosphates by rat liver microsomes. Each tube contained in 1 ml, 2,500 cpm of C^{14}-allyl pyrophosphates (1,900 cpm, i.e., 0.024 μmole in farnesyl pyrophosphate); 5 mM $MgCl_2$, 0.1 M buffer, and 0.05 ml microsomes. Incubation under air at 37° for 1 hour. Extent of hydrolysis measured by appearance of free C^{14}-prenols.

respectively, are converted into squalene (9, 11), no synthesis of C^{14}-squalene or of C^{14}-allyl pyrophosphates could be demonstrated (Table 5). The free prenols are obviously not precursors of squalene, and the hydrolysis of the allyl pyrophosphates by microsomes is an irreversible process.

Conversion of C^{14}-Prenols into Acids by Liver Soluble Enzymes; Properties of the Enzymes. Free C^{14}-prenols were shown to be largely converted into acids in the presence of soluble enzymes (Table 5, incubation Nos. 3–5). The extent of the transformation was independent of the presence or absence of TPNH or of ATP. When the substrate was a prenol preparation consisting mostly of C^{14}-nerolidol, only about 20% of the total counts appeared in the acidic fraction (Table 5, incubation No. 6). This proportion of the counts corresponded to the primary C^{14}-prenol content of the substrate preparation. Clearly, only primary alcohols, as might be expected, could act as precursors of the acids.

It was shown in further experiments, which need not be detailed, that neither microsomes nor Mg^{++} were needed for the conversion of prenols into acids, but only the enzymes present in the F^{60}_{30}-protein fraction of the liver homogenates. Phosphate or tris-HCl buffers of the same pH were equally suitable for studying the transformation of the alcohols into acids.

As it appeared most probable that some dehydrogenase(s) were concerned in the conversion of the prenols into acids, it was somewhat intriguing that no definite need for a pyridine nucleotide could be demonstrated at first, in spite of the fact that the F^{60}_{30}-preparations were always dialyzed. As it was suspected that pyridine nucleotides were too firmly bound to the proteins to be removed by dialysis, a preparation of F^{60}_{30}-enzymes (5 ml; 39 mg protein/ml) was treated with about 1 g of Dowex-1 ion exchange resin for 15 minutes according to Tietz and Popják (16). This treatment depressed the conversion of C^{14}-prenols to acids by 30%, but the addition of DPN to the incubation completely overcame the depression and resulted in a 12% increase in the yield of acids as compared to that observed with untreated F^{60}_{30}-enzymes.

Figure 3 illustrates that the conversion of C^{14}-prenols into acids by a dialyzed F^{60}_{30}-preparation is fairly rapid, about 28% and 70% of the added substrate having been converted into acids in 2 and 15 minutes, respectively. The initial rate of this conversion could be increased to 42% in 2 minutes by the addition of DPN (0.33 μmole), and by ensuring rapid reoxidation of any DPNH formed by the use of pyruvate and lactic dehydrogenase.

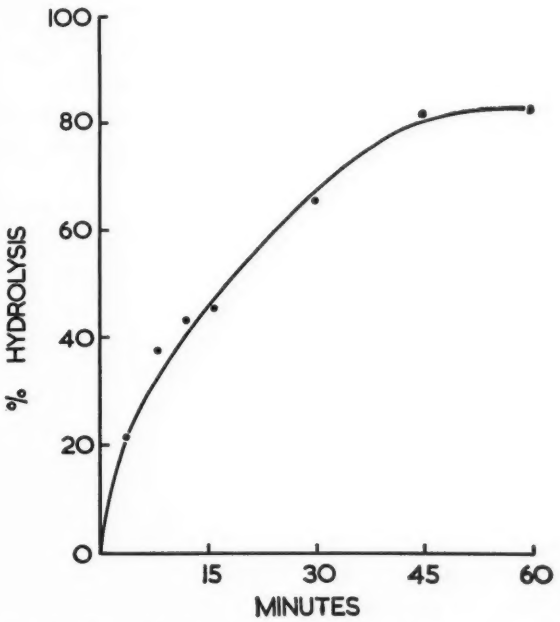


FIG. 2. Rate of hydrolysis of C^{14}-allyl pyrophosphates by rat liver microsomes at pH 7.4. Conditions of incubations as for Figure 1, except that tris-HCl buffer was used.

TABLE 5. FORMATION OF PRENOIC ACIDS FROM C^{14}-PRENOLS BY SOLUBLE ENZYMES OF RAT LIVER. FAILURE OF SYNTHESIS OF ALLYL PYROPHOSPHATES AND OF SQUALENE FROM PRENOLS*

Incubation No.	Additions	C^{14} Found in			
		Squalene	Free Prenols	Prenoic Acids	Allyl-PP
		cpm	*cpm*	*cpm*	*cpm*
1†	TPNH	1806	399	100	840
2‡	TPNH	0	5733	600	90
3‡	F_o^{60}, TPNH, ATP	0	347	6640	20
4‡	F_o^{60}, ATP	0	651	6900	30
5‡	F_o^{60}	0	777	6500	20
6§	F_o^{60}, TPNH, ATP	0	4158	1020	80

* Each tube contained in a final volume of 1 ml, 0.1 M K-phosphate buffer, pH 7.4; 5 mM $MgCl_2$; 30 mM nicotinamide; 10 mM NaF; and 0.05 ml of microsome suspension. Additions of F_o^{60}-enzymes (14 mg), of TPNH (1 μmole, generated with 2 μmoles of glucose-6-phosphate and glucose-6-phosphate dehydrogenase), and of ATP (7.5 μmoles) were made as shown. Incubations under N_2 for 1 hour at 37°.

† Substrate, C^{14}-allyl pyrophosphates: 3,750 cpm, of which 2,800 cpm (0.036 μmole) were in farnesyl pyrophosphate.

‡ Substrate, C^{14}-prenols: 7,500 cpm, of which 5,600 cpm (0.072 μmole) were in *trans-trans*-farnesol.

§ Substrate, C^{14}-prenols: 6,500 cpm, of which 4,200 cpm (0.054 μmole) were in nerolidol, and 1,300 cpm (0.017 μmole) in farnesol.

The enzymes responsible for the conversion of prenols into acids had a broad pH optimum lying between 7.2 and 8.2 (Fig. 4).

The dehydrogenation of C^{14}-prenols was almost completely inhibited (97%) by 1 mM PCMB. This inhibition was partially reversed (47%) in the presence of a small excess of GSH (1.5 mM). Other reagents for thiol groups, when tested in 1 mM concentration, were found to be inhibitory to a variable extent: N-ethyl maleimide gave a 40%, iodoacetamide a 22%, and iodoacetate only a 7% inhibition. BAL (2 mM) was also inhibitory (36%), and the inhibition caused by it was augmented to 72% by 1 mM sodium arsenite, which by itself was without effect. Silver and copper ions (0.5 mM) stopped completely the dehydrogenation of prenols. Much of the activity of F_{30}^{60}-preparations was lost on storage at −15° for more than 1 month, but it could be largely restored by the addition of 2 to 4 mM GSH.

Identification of the Prenoic Acids Derived from Mevalonate or from Prenols. The C^{14}-prenols and prenoic acids synthesized from mevalonate-2-C^{14} were identified by gas-liquid radiochromatography (4). Popják and Cornforth (14) have also reported on the behavior of the prenols and of the prenoic acids in gas-liquid chromatography, which allows the identification of these substances with confidence, provided the analyses are made both on columns with polar and nonpolar station-

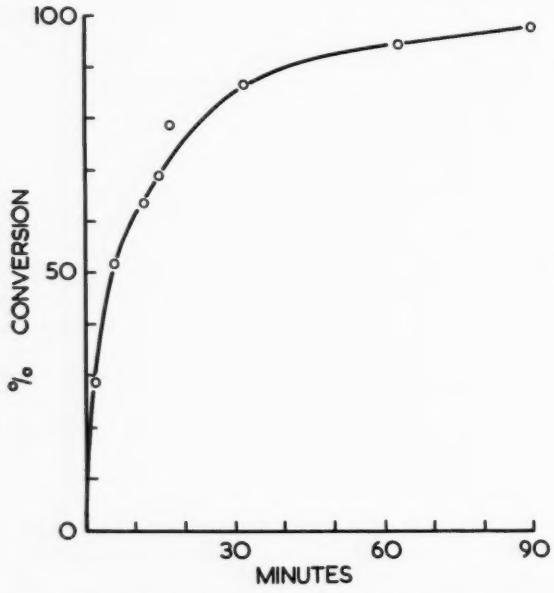


FIG. 3. Rate of conversion of C^{14}-prenols into acids by rat live soluble enzymes. Each tube contained in 1 ml, 2,200 cpm of C^{14}-prenols (1,900 cpm = 0.025 μmole of farnesol + geraniol); F_{30}^{60}-enzymes (15 mg protein); and 0.1 M phosphate buffer pH 7.4. Incubation under N_2 at 37°.

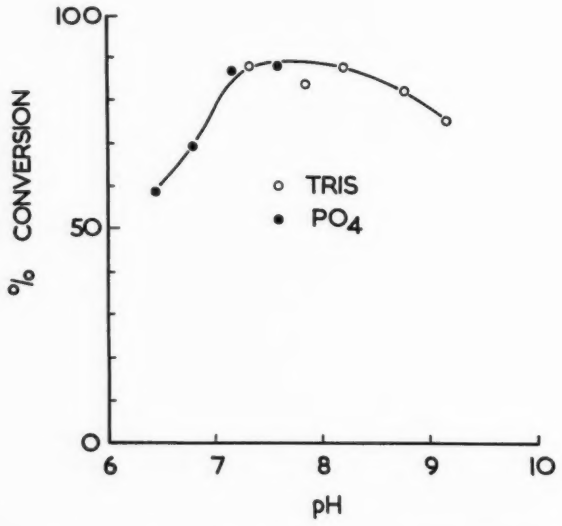


FIG. 4. Relation between pH and conversion of C^{14}-prenols into acids by rat liver soluble enzymes. Each tube contained in 1 ml, 2,500 cpm of C^{14}-prenols, of which 2,100 cpm were in farnesol and geraniol; F_{30}^{60}-enzymes (33 mg protein); and 0.1 M buffer. Incubation under air at 37° for 30 minutes.

ary phases. It was recognized in this laboratory some time ago (2, 3) that one of the main acids derived from mevalonate-2-C^{14} in liver homogenates was *trans-trans*-C^{14}-farnesoate (Fig. 6 *III*). The retention volume of the methyl ester of this acid was identical with that of the *trans-trans* component of synthetic methyl farnesoate. Furthermore, it was shown[4] that after catalytic hydrogenation of the above C^{14}-acid, it became inseparable by gas-liquid chromatography from an authentic specimen of methyl 3,7,11-trimethyldodecanoate, which is the saturated analogue of methyl farnesoate. Also, reduction of the methyl ester with $LiAlH_4$ in ether at $-30°$ (14) gave *trans-trans*-C^{14}-farnesol.

Prenoic Acids with Retention Volumes Smaller than That of Farnesoate. In order to study these compounds more closely, a pooled specimen of C^{14}-prenoic acids was separated by gas-liquid chromatography at 197° into fractions emerging from the column before and after *trans-trans*-farnesoate. The fraction containing substances with the smaller retention volumes was then analyzed by gas-liquid radiochromatography at 100° on a column with ethylene glycol-adipic acid polyester stationary phase. By this procedure it was possible to compare more accurately the retention volumes of the radioactive methyl esters with those of internal (unlabeled) standards chromatographed simultaneously. We have identified in this way the methyl

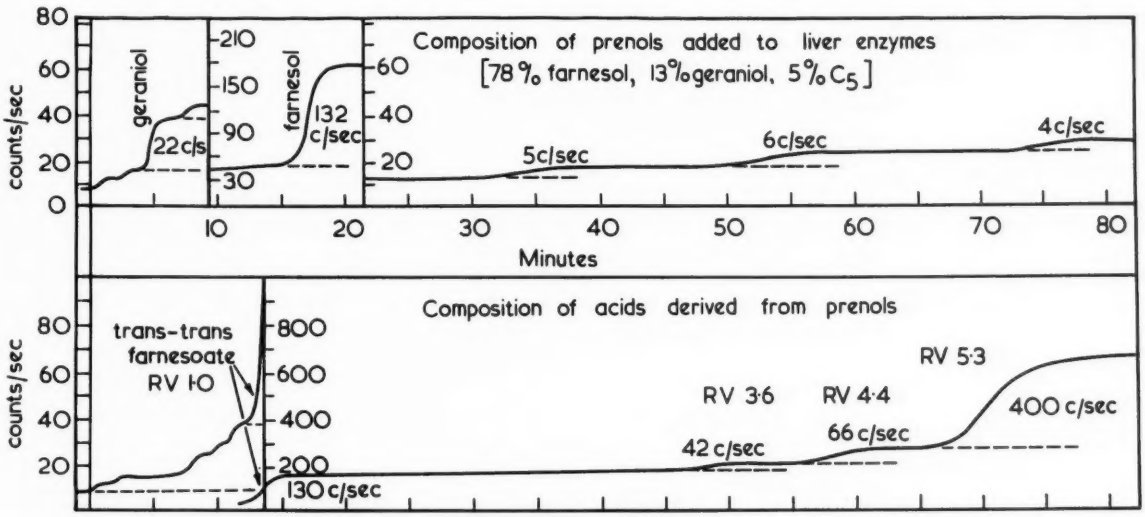


FIG. 5. Gas-liquid radiochromatographic analysis of C^{14}-prenols and acids derived from them. (*a*) C^{14}-prenols added to rat liver soluble enzymes; (*b*) C^{14}-acids derived from the C^{14}-prenols after 1-hour incubation at 37°. Four-foot column with ethylene glycol-adipic acid polyester stationary phase; 197°.

Gas-liquid radiochromatographic analysis of the C^{14}-acids derived from mevalonate-2-C^{14}, from C^{14}-allyl pyrophosphates, or from the primary C^{14}-prenols revealed identical components, and showed that in addition to farnesoate, other radioactive acids were also formed. Some of these had retention volumes smaller than that of farnesoate, and several emerged from the columns much later than farnesoate. Figure 5 is a typical record of the analysis of the C^{14}-methyl esters obtained by the incubation of C^{14}-prenols with F_{30}^{60}-enzymes. Table 6 summarizes the gas-liquid chromatographic analyses of the prenoic acids in eight experiments, and shows that no less than 13 acidic compounds were encountered.

[4] In collaboration with Mrs. M. Horning of the National Heart Institute, National Institutes of Health, Bethesda 14, Md.

esters of C^{14}-3,3-dimethylacrylate (Fig. 6 *I*), of C^{14}-*cis*-geranoate, and of C^{14}-*trans*-geranoate (Fig. 6 *II*).

Prenoic Acids with Retention Volumes Greater than That of Farnesoate. It was apparent from a balance of the counts in the acidic fractions and of the C^{14}-prenols used as substrates that the radioactive methyl esters with a retention volume greater than that of farnesoate could have been derived only from *trans-trans*-C^{14}-farnesol (Fig. 5). It was necessary to investigate whether these compounds occurred as natural enzymic products, or were artifacts produced during the extraction procedure. To check the latter possibility, a pool of the methyl esters of C^{14}-prenoic acids was subjected to gas-liquid chromatography, and the fraction corresponding to methyl *trans-trans*-C^{14}-farnesoate collected. This sample of 95% pure *trans-trans-*

C^{14}-farnesoate was then treated in the usual way, i.e., it was saponified with ethanolic potassium hydroxide, then acidified with HCl, extracted with petroleum ether, and methylated again. Radiochromatography showed that over 60% of the *trans-trans*-C^{14}-farnesoate had been transformed into a C^{14}-acid with a retention volume 5.78 times greater than that of the original compound. A similar phenomenon could be observed with a mixture of unlabeled *trans-trans* and *cis-trans*-farnesoate (Table 6). These results indicated that the extraction procedure was responsible for the conversion of farnesoate into compounds with a much larger retention volume. The nature of these compounds is not known but it is suspected that the one with retention volume 5.78 times that of farnesoate might be the monocyclic derivative (Fig. 6 *V*) of farnesoic acid (17, 18). It was also considered that these substances might be hydroxylated derivatives of farnesoic acid; however, attempted acetylation with acetic anhydride in pyridine did not change the retention volume of any of the radioactive fractions. Saponification, etc., of the methyl esters of *cis*-geranoic and *trans*-geranoic acids likewise resulted in a partial transformation of the acids into substances with retention volumes larger than those of the original methyl esters (Table 6). These esters had retention volumes very close to that of methyl *trans-trans*-farnesoate, and the substance derived from methyl *trans*-geranoate was indistinguishable in gas-liquid chromatography from a specimen of *β-cyclo*-geranoate (Fig. 6 *VI*).

TABLE 6. GAS-LIQUID CHROMATOGRAPHY OF METHYL C^{14}-PRENOATES*

Retention Volume of Prenoates		Fraction Identified as	Distribution of Radioactivity in Fractions Relative to *trans-trans*-Farnesoate
Relative to Methyl Stearate	Relative to *trans-trans*-Farnesoate		
0.02	0.03	3,3-Dimethylacrylate	0.07
0.09	0.15	*cis*-Geranoate	0.25
0.12	0.20	*trans*-Geranoate	0.31
0.32	0.54	Unidentified	0.85
0.45	0.78	*cis-trans*-Farnesoate	0.13†
0.54	0.94	*cis*-Geranoate derivative	—
0.59	1.00	*trans-trans*-Farnesoate	1.00
0.65	1.12	*β-cyclo*-Geranoate	—
0.80	1.36	Unidentified	0.14†
1.30	2.21	Unidentified	0.20†
2.20	3.74	Unidentified	0.07
2.76	4.78	*cis-trans*-Farnesoate derivative	0.69
3.34	5.78	*trans-trans*-Farnesoate derivative	1.91
3.60	6.12	Unidentified	0.24†
4.90	8.33	Bi-*cyclo*-farnesoate	0.54†

* Derived from C^{14}-prenols, or from mevalonate-2-C^{14}. Gas-liquid chromatography on ethylene glycol-adipic acid polyester columns at 197°. Mean values derived from eight experiments.
† Fractions not always present.

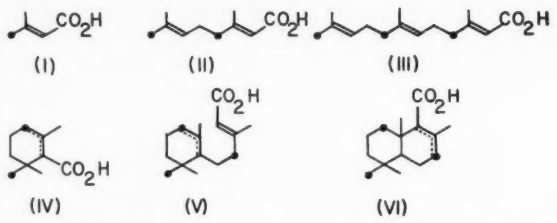


FIG. 6. Prenoic acids biosynthesized from mevalonate-2-C^{14} (*I–III*), and cyclized forms of geranoic and farnesoic acids (*IV–VI*). Positions marked with closed circles are those derived from C-2 of mevalonate.

Table 6 shows that the origin of some acids is still unidentified, but we are inclined to believe that the principal compounds, formed enzymically, are the mono-, di-, and triprenoic acids, and that most of the others are probably derived from the latter two by cyclization and isomerization during the isolation procedures.

As a further check on the origin of the C^{14}-acids, derived ultimately from mevalonate-2-C^{14}, we have studied the conversion of unlabeled geraniol and farnesol into acids by the F^{60}_{30}-enzymes. The specimen of geraniol was 98% pure and contained only 2% nerol. The preparation of "farnesol" contained 56% *trans-trans*-farnesol, 32% *cis-trans*-farnesol, and 12% nerolidol. As our aim was to obtain maximum yields of acids for gas-liquid chromatographic analysis, the DPNH generated during prenol dehydrogenation (see below) was oxidized continuously to DPN by the addition of the substrates (ADP and PEP) for the pyruvic kinase-lactic dehydrogenase system, which is present in abundance in our F^{60}_{30}-preparations. Two incubations of 10 ml each were set up containing the following: 0.1 M K-phosphate buffer, pH 7.4; F^{60}_{30}-enzymes, 19 mg protein per ml; DPN, 0.5 mM; ADP, 1.5 mM; PEP, 1.3 mM; $MgCl_2$, 5 mM; and geraniol, 1.4 mM in one experiment, and "farnesol," 2 mM in the other. The incubations at 37° lasted for 85 minutes. In the first experiment practically all the geraniol was converted into *trans*-geranoic acid and into the substance thought to be β-cyclogeranoate (Fig. 6 *IV*), both having been identified by gas-liquid chromatography.

In the experiment with "farnesol" only 17% of the total amount of substrate was converted into acid, but

all of this was derived entirely from the *trans-trans*-farnesol component of the substrate; the *cis-trans*-farnesol and nerolidol were recovered unchanged. Gas-liquid chromatography of the methyl esters of the acids formed showed these to consist of *trans-trans*-farnesoate and of a substance with a retention volume of 5.78 times that of methyl *trans-trans*-farnesoate.

The Role of Liver Alcohol Dehydrogenase and Aldehyde Dehydrogenase in the Origin of Prenoic Acids. It seemed most probable to us that liver alcohol dehydrogenase was involved in the transformation of the prenols into acids. In order to test this idea it was necessary to ascertain first whether the prenols could act as substrates for alcohol dehydrogenase, as no information was available on the subject. Table 7 shows that the rates

TABLE 7. SUBSTRATE SPECIFICITY OF HORSE LIVER ALCOHOL DEHYDROGENASE *

Alcohol	Initial Rate† $\Delta E_{340}/10$ Minutes
3-Methylbut-3-en-1-ol (isopentenol)	0.44
3,3-Dimethylallyl alcohol	0.29
Geraniol	0.40
Nerol (*cis*-geraniol)	0.19
trans-trans-Farnesol	0.21
cis-trans-Farnesol	0.15
Potassium mevalonate	0.00
Allyl alcohol	0.58
isoAmyl alcohol	0.24
Pentan-2-ol	0.05
Ethanol	0.37

* Each cuvette contained in 3 ml, 0.1 M NaOH-glycine buffer, pH 9.5; crystalline horse liver alcohol dehydrogenase, 15 μg; 0.2 mM DPN; and 3.33 mM alcohol. The reaction was started by the addition of the alcohol, and the course of DPN reduction followed at 340 mμ and at 37°, using cuvettes with 1-cm light path.

† The initial rate of DPN reduction was calculated from the extrapolation of the linear part of the light-extinction curve to 10 minutes. A change of 0.1 in the value of E_{340} is equivalent to the reduction of 0.048 μmole of DPN under our conditions.

of dehydrogenation of isopentenol, dimethylallyl alcohol, geraniol, and farnesol by liver alcohol dehydrogenase compare favorably with that of ethanol. Nerol (*cis*-geraniol) and *cis-trans*-farnesol were dehydrogenated at a slower rate than their *trans*-isomers. The rate of dehydrogenation of a few other alcohols is also shown in Table 7. As was to be expected, the tertiary alcohols, *d*-linalool and nerolidol, were completely unreactive. DL-Mevalonate (as the potassium salt) was also inert toward the alcohol dehydrogenase. Yeast alcohol dehydrogenase was tested for specificity of substrates under the same conditions as the horse liver enzyme, and although it showed the expected activity toward ethanol (initial rate of dehydrogenation gave a $\Delta E_{340}/10$ minutes of 0.58), it was completely unreactive with isopentenol, dimethylallyl alcohol, geraniol, and farnesol.

As is well known, the rate of dehydrogenation of ethanol with liver alcohol dehydrogenase is faster at pH 9.5 than at pH 7.4. The initial rate of the dehydrogenation of geraniol was the same at these two pH values, while with dimethylallyl alcohol the initial rate of dehydrogenation was faster at pH 7.4 than at pH 9.5 ($\Delta E_{340}/10$ minutes = 0.47 at pH 7.4, against $\Delta E_{340}/10$ minutes = 0.29 at pH 9.5).

In experiments designed to study the equilibria of the dehydrogenase reaction (see under Methods), it was found that at pH 7.4 the reduction of DPN in the presence of ethanol was barely detectable, a fact that was not unexpected since equilibrium is known to be greatly in favor of aldehyde reduction (19). In contrast, dimethylallyl alcohol and geraniol were quite efficiently dehydrogenated (Fig. 7*b*). Assuming that the amount of alcohol dehydrogenated was equal to the amount of DPN reduced, the values for the equilibrium constant at 37°, at pH 7.4

$$\left[K_{eq} = \frac{(\text{aldehyde})\ (\text{DPNH})\ (H^+)}{(\text{alcohol})\ (\text{DPN})}\right]$$

were 1.1×10^{-12} for ethanol; 2.8×10^{-10} for dimethylallyl alcohol, and 4.4×10^{-11} for geraniol.

Spectrophotometric experiments, similar to those described for crystalline liver alcohol dehydrogenase, were also done with the F_{30}^{60}-enzymes and the various alcohols. The F_{30}^{60}-enzymes were very active in reducing DPN (but not TPN) in the presence of prenols, and the initial rate of DPN reduction was proportional to enzyme concentration in the range studied (0.5 to 4 mg protein/ml). When experiments similar to those illustrated in Figure 7*b* were reproduced with F_{30}^{60}-enzymes (1.5 mg/ml), it was found that, as with crystalline alcohol dehydrogenase, both the initial rate and the extent of the dehydrogenation of dimethylallyl alcohol and of geraniol were greater than those of ethanol (Fig. 7*a*). However, for each alcohol tested (including ethanol), more DPN was reduced than in the corresponding experiment with crystalline alcohol dehydrogenase alone. The different levels of the equilibrium attained during dehydrogenation of prenols by crystalline liver alcohol dehydrogenase and by the F_{30}^{60}-enzymes are clearly seen by comparing Figures 7*a* and 7*b*. An accurate determination of the amount of DPN produced by the alcohols with F_{30}^{60}-enzymes was difficult because of a slow reoxidation of the DPNH generated. This phenomenon was more

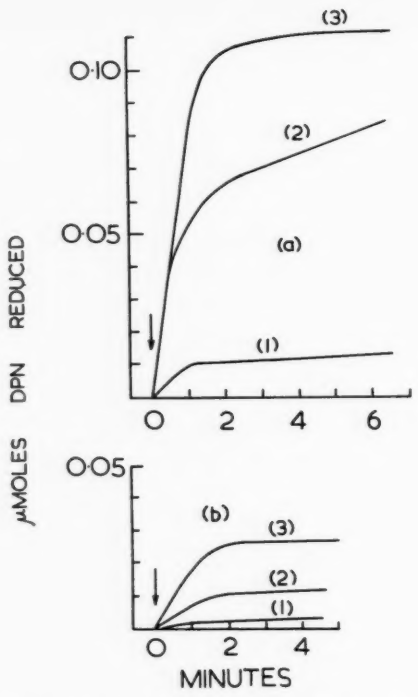


FIG. 7. Dehydrogenation of alcohols by horse liver alcohol dehydrogenase and by rat liver soluble enzymes. The curves represent the formation of DPNH as a function of time after the addition of 0.1 µmole of alcohol. Each cuvette contained in 3 ml, 0.5 mM DPN, 0.1 M phosphate buffer pH 7.4, and liver alcohol dehydrogenase (25 µg/ml) in (*b*); or F_{30}^{60}-enzymes (1.5 mg protein/ml) in (*a*). Curves (*1*): reaction with ethanol; curves (*2*): reaction with geraniol; and curves (*3*): reaction with dimethylallyl alcohol.

pronounced at pH 9.5 than at pH 7.5, and was due to a DPNH-oxidase present in the F_{30}^{60}-enzymes. In one instance the course of DPN reduction in the presence of dimethylallyl alcohol was corrected for this DPNH oxidation by observing the rate of oxidation of DPNH added to a control cuvette. It was calculated that 0.16 µmole of DPN was reduced by 0.1 µmole of dimethylallyl alcohol.

Since it is reasonable to assume that the equilibrium position of prenol dehydrogenated by horse liver alcohol dehydrogenase is similar to that catalyzed by rat liver alcohol dehydrogenase, the reduction of nearly 2 moles of DPN per mole of prenol in the F_{30}^{60}-preparations strongly suggests the participation of a second dehydrogenase. We suggest that this second enzyme is an aldehyde dehydrogenase which catalyzes the irreversible dehydrogenation to acids of the aldehydes generated from the prenols by liver alcohol dehydrogenase. Certainly, we could not demonstrate a reduction of 3,3-dimethylacrylate with DPNH and F_{30}^{60}-enzymes. On the other hand, when unlabeled farnesal (73% *trans-trans* + 27% *cis-trans* isomer, 1.2 µmoles) was incubated at pH 7.4 with F_{30}^{60}-enzymes (100 mg in 4 ml) for 1 hour at 37°, the *trans-trans*-isomer (but not the *cis-trans*-isomer) was converted into *trans-trans*-farnesoic acid, which was identified by gas-liquid chromatography.[5]

Synthesis of Allyl Pyrophosphates, Prenols, and Prenoic Acids in Vivo. In order to decide whether the prenoic acids found under the highly artificial conditions of the *in vitro* experiments had any physiological significance, we gave 5.4 µmoles (2.5×10^6 cpm) of DL-mevalonate-2-C^{14} by stomach tube to a 22-g mouse. The animal was killed 70 minutes later, and the liver analyzed for allyl pyrophosphates, free prenols, prenoic acids, squalene, and for cholesterol, by the same procedures as described for the enzyme incubations. Of the injected dose of DL-mevalonate, 2.35% (or 4.7% of the biologically active [+] isomer) was found incorporated into various components of the liver. Expressed as a percentage of total counts found in the liver, 11% was found in sterol digitonides, 46% in squalene, 4% in allyl pyrophosphates, 16% in free prenols, and 23% in prenoic acids. Upon gas-liquid radiochromatography, the main component of the prenoic acids was identified as *trans-trans*-farnesoate; *trans-trans*-farnesol was the main constituent of the prenols.

DISCUSSION

Evidence has been presented that in a liver enzyme system, mevalonate is a precursor not only of squalene but also of certain prenoic acids. These acids are formed by a mechanism completely different from that of the synthesis of normal fatty acids. This mechanism consists of the dephosphorylation of allyl pyrophosphates (intermediates in squalene biosynthesis) and subsequent dehydrogenation of the resulting prenols by liver alcohol dehydrogenase and aldehyde dehydrogenase into the corresponding acids. The reason why the synthesis of prenoic acids has not been reported from laboratories studying squalene biosynthesis with yeast enzymes is most probably due to the fact that yeast alcohol dehydrogenase is totally inactive toward prenols. A similar observation had already been made by Bliss (20) with vitamin A.

The microsomal phosphatase, which hydrolyzes the allyl pyrophosphates, has properties somewhat

[5] The retention volume of *trans-trans*-farnesal is very close to that of *trans-trans*-farnesol on both ethylene glycol-adipic acid polyester columns and on Apiezon-L columns at 197°, but is widely different from the retention volume of methyl *trans-trans*-farnesoate.

different from other phosphatases; its pH optimum is quite different from that of serum alkaline phosphatase, but is close to those of three other phosphatases present in microsomes: glucose-6-phosphatase (21), 5′-nucleotidase (22), and phosphatidic-acid phosphatase (23). Whether or not the phosphatase activity of the microsomes studied by us is specific for the allyl pyrophosphates cannot be decided, as these compounds are hydrolyzed by a variety of phosphatases, such as intestinal alkaline phosphatase, prostatic acid phosphatase, and snake venom phosphatases (9). Contrary to the needs of some phosphatases (e.g., alkaline phosphatase or snake venom phosphatases), the microsomal enzyme needs no Mg^{++} for activation, and chelating agents or sulfhydryl inhibitors had no effect on it. The inhibition of the microsomal phosphatase by bovine serum albumin was surprising; the mechanism of action of the albumin is probably indirect and may be attributed to a binding of the allyl pyrophosphates to this protein. It was found in experiments, not recorded here, that potassium farnesoate and the mono-, di-, and triprenols, in concentrations of 0.4 to 2 mM, could partially reverse the inhibition caused by albumin. Possibly these substances and the allyl pyrophosphates were competing for identical binding sites on the albumin. It is of interest to point out that the microsomes also contain the squalene synthetase system (9) which converts two molecules of farnesyl pyrophosphate into squalene, but only in the presence of a reduced pyridine nucleotide, TPNH or DPNH. In the absence of these coenzymes the squalene synthetase system is completely ineffective, and the action of the microsomal phosphatase comes to the fore, destroying the substrate for the squalene synthetase, as free prenols cannot be utilized for the synthesis of squalene.

The experimental evidence presented on the dehydrogenation of prenols to acids indicates that this occurs in two steps, with aldehydes as intermediates, involving both an alcohol dehydrogenase and an aldehyde dehydrogenase. We have not examined the possibility that the acids may arise from the prenols by the action of the liver alcohol dehydrogenase alone, through a dismutation of the aldehydes to acid and alcohol (24). It does not appear, however, to be a very likely mechanism for the formation of prenoic acids, since spectrophotometric studies with the F_{30}^{60}-enzymes showed the reduction of nearly 2 moles of DPN by 1 mole of prenol.

It is well known that both the alcohol and aldehyde dehydrogenases of liver contain SH-groups at their active sites (25). The inhibition of the dehydrogenases present in our F_{30}^{60}-preparations by PCMB, N-ethylmaleimide, iodoacetamide, Ag^{+}, and Cu^{++} suggests very strongly that these two dehydrogenases are responsible for the formation of the prenoic acids. The failure of iodoacetate to inhibit the conversion of prenols to acids by the F_{30}^{60}-preparations is in harmony with the observation that liver alcohol dehydrogenase is insensitive to this reagent (26). The sensitivity of the F_{30}^{60}-dehydrogenases to PCMB was such that PCMB was used in preparative incubations when C^{14}-prenols were being made from C^{14}-allyl pyrophosphates in the presence of both microsomes and F_{30}^{60}-enzymes (see under Methods). The use of PCMB in such incubations depended also on the fact that the microsomal phosphatase was completely insensitive to this reagent.

Our finding, that the enzymes converting prenols into acids are confined largely to the F_{30}^{60}-fraction of the soluble supernatant of liver homogenates, is also consistent with the properties of liver alcohol and aldehyde dehydrogenases, which are known to be precipitated with $(NH_4)_2SO_4$ between 55% and 75%, and 51% and 60% saturation, respectively (25, 27).

Spectrophotometric experiments with the F_{30}^{60}-enzymes showed clearly that the dehydrogenation of prenols was a DPN-linked reaction. The observation that the C^{14}-prenols could be converted into acids by the F_{30}^{60}-enzymes, even in the absence of added DPN, can be readily explained by the assumption that the enzyme preparation contained an amount of bound DPN sufficient for the dehydrogenation of the small quantities of C^{14}-prenols (0.01 to 0.03 μmole) used in the experiments. The DPN-oxidase present in the F_{30}^{60}-preparations would ensure, moreover, that the DPNH generated is reoxidized to DPN. Hence, for the dehydrogenation of 0.01 to 0.03 μmole of prenols, even catalytic amounts of DPN would suffice. The firm binding of DPN to the apo-enzyme in alcohol dehydrogenase is well known. The above assumption is supported by the finding that treatment of the F_{30}^{60}-preparation with Dowex-1 ion exchange resin caused some inactivation, which could be fully compensated by the addition of DPN.

The irreversible DPN-linked dehydrogenation of prenols to acids by F_{30}^{60}-enzymes is also in harmony with our assumption that aldehyde, as well as alcohol dehydrogenase, is involved in the process. Racker (28) observed that acetate could not be reduced to acetaldehyde with DPNH and liver aldehyde dehydrogenase. Thus the quantitative conversion of prenols to acids, which we have observed, could be explained only by the participation of two enzymes, since the equilibrium of the reaction with prenols catalyzed by alcohol dehydrogenase alone was always in favor of the alcohol.

The presence of aldehyde dehydrogenase, on the other hand, would drive the reaction toward complete dehydrogenation.

The finding that the prenols are highly specific substrates for liver (but not yeast) alcohol dehydrogenase suggests to us that the physiological role of this enzyme is connected primarily with the metabolism of intermediates of sterol biosynthesis and of similar natural products, and only fortuitously with dealing with man's social habit of consuming ethanol.

Evidence has been accumulated that the prenoic acids are 3,3-dimethylacrylic, *trans*-geranoic, and *trans-trans*-farnesoic acids, together with artifacts produced from these during extraction procedures. We have had no evidence for the presence of hydroxy acids. The dehydrogenases converting the prenols into acids appear to be specific for the *trans*-isomers of alcohols, as no formation of *cis-trans*-farnesoic acid from *cis-trans*-farnesol could be observed. The small amounts of the *cis*-isomers of C^{14}-geranoic acid, and of *cis-trans*-C^{14}-farnesoic acid derived from the C^{14}-prenols, are believed to arise through isomerization during the extraction procedure. The transformation of both geranoic and farnesoic acids into what we believe to be their cyclized forms under the relatively mild conditions of our extraction procedure appears somewhat surprising, since the acid catalyzed cyclization of these substances usually requires much more vigorous conditions (17, 18).

In some earlier communications from this laboratory (2, 3) it was stated that radioactive "terpenoid" acids were formed in liver homogenates, not only from mevalonate-2-C^{14} but also from mevalonate-1-C^{14}. This was a genuine observation in the sense that the particular batch of mevalonate-1-C^{14} gave rise to C^{14}-prenoic acids. One of us (Popják) repeated these earlier experiments with a new batch of mevalonate-1-C^{14} and failed to find an incorporation of C^{14} into prenoic acids: the samples contained only a few insignificant counts. It is suspected that the original batch of mevalonate-1-C^{14} may have contained label on some position other than the carboxyl carbon.

Synthesis of prenoic acids from mevalonate is not restricted to the highly artificial conditions of incubations of liver homogenate or of its fractions. Unpublished observations in this Unit by K. Fletcher and N. B. Myant showed that the same prenoic acids as those found in homogenates occurred in liver slices metabolizing mevalonate. Elwood *et al.* (29) reported recently that for every 22 atoms of C^{14} incorporated into cholesterol from mevalonate-2-C^{14} by liver slices, 1 atom of C^{14} appeared in the fatty acid fraction. Garattini *et al.* (30) have found that 2 hours after the intraperitoneal injection of mevalonate-2-C^{14} into 12-day-old rats, the radioactivity recovered in liver fatty acids was as great as that recovered in liver cholesterol, but the radioactive acids have not been identified. Our experiment with the mouse showed that the radioactivity found in liver fatty acids was associated mainly with *trans-trans*-farnesoate.

The physiological role of the prenoic acids is at present a matter of conjecture. Clearly, they represent an alternative pathway for the metabolism of the allyl pyrophosphates, and consequently may be involved in the regulation of cholesterol synthesis in two ways. The effect of the hydrolysis of the allyl pyrophosphates to free prenols by itself is to decrease the amount of substrate available for sterol synthesis. A further possibility is that the prenoic acids also exert an inhibitory action on various enzymic reactions of cholesterol biosynthesis. Wright and Cleland (31) were the first to observe an inhibition of cholesterol biosynthesis from mevalonate by farnesoic acid in liver homogenates, and this was confirmed in our laboratory. It was also shown by Levy and Popják (13) that farnesoate inhibited mevalonic kinase, and by Popják *et al.* (32) that some analogues of farnesoic acid were quite powerful inhibitors of cholesterol synthesis from mevalonate *in vitro*. In spite of these highly suggestive results it remains for future work to determine precisely the function and metabolism of prenoic acids *in vivo*.

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Effect of mevalonate analogues upon cholesterol biosynthesis

HERBERT WEISS,* ELLIOTT SCHIFFMANN, and ELWOOD TITUS

Laboratory of Chemical Pharmacology, National Heart Institute, National Institutes of Health, Bethesda 14, Maryland

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SUMMARY

Three analogues of mevalonic acid have been found to inhibit cholesterol biosynthesis in liver homogenates in the following order of efficacy: 3-methyl-3-hydroxy pentanoic acid > Δ^2-3-methyl pentenoic acid > Δ^3-3-methyl pentenoic acid. The hydroxy acid produces half the maximum inhibition at a concentration of 5 μmoles/ml of homogenate. This corresponds to a ratio of 100 μmoles of inhibitor to 1 μmole of substrate.

The presence of cholesterol-containing plaques in atherosclerotic lesions has stimulated studies of compounds that inhibit the biosynthesis of the sterol. Blohm and MacKenzie (1) have reported that the oral administration of Triparanol (MER-29), whose site of action has recently been determined (2), lowers both serum and tissue levels of cholesterol.

Major efforts in the search for compounds which antagonize cholesterol biosynthesis have been undertaken as a result of work showing that isoprenoids are intermediates in cholesterol formation. Thus, when β,β′-dimethyl acrylyl-CoA was considered at one time to be a major isoprenoid intermediate, analogues of this compound were tested both *in vivo* (3, 4) and *in vitro* (5) for their effect upon cholesterol biosynthesis. The demonstration that mevalonic acid (6) is an important precursor of isoprenoid units had led to the testing of analogues of this compound.

Stewart and Woolley (7), Tamura *et al.* (8), and Wright (9) have screened a variety of compounds as mevalonate antagonists in certain lactobacilli which require the acid for growth.

Using rat liver homogenates, Singer *et al.* (10) have shown that fluoromevalonic acid inhibits the formation of cholesterol from both C^{14}-labeled acetate and mevalonic acid.

Mentzer *et al.* (3) have reported that liver cholesterol levels in rats are lowered by 30% after the oral administration of Δ^3-3-methyl pentenoic acid while the Δ^2 isomer is ineffective. These compounds are structurally similar to β,β′-dimethylacrylic acid.

Gey *et al.* (5) have found that both the Δ^3 compound and Δ^4-3-methyl-3-hydroxy-pentenoic acid reduce the incorporation of C^{14}-acetate into cholesterol in rat liver homogenate.

Supniewski *et al.* (4) studied the effect of the Δ^3 compound in pigeons maintained on an atherosclerogenic diet. The intramuscular administration of this material reduced aortic lesions which had developed during cholesterol feeding. Given together with the atherosclerogenic diet, the compound prevented the formation of sclerotic nodules.

The reportedly greater effectiveness of the Δ^3 acid compared to the Δ^2 acid and the structural similarity of the Δ^3 acid to mevalonic acid were considerations which led to the present work. These efforts involved a study of the inhibitory effects upon cholesterol biosynthesis from mevalonic acid-2-C^{14} *in vitro* by three compounds: the Δ^2 acid, Δ^3 acid, and 3-methyl-3-hydroxy-pentanoic acid. We have found that the hydroxy acid is the most effective compound.

METHODS

Materials. Digitonin was obtained from Fisher Scientific Co.; ATP and DPN were purchased from Sigma Chemical Co. The dibenzylethylenediamine salt of mevalonic acid-2-C^{14} was obtained from Isotope Specialties Co. The compounds tested for inhibitory properties were prepared by synthetic procedures to be described.

Methods. The amine salt of C^{14}-mevalonate was converted to an aqueous solution of potassium mevalonate as follows: An aqueous solution of the amine

* Deceased August 20, 1960.

salt was adjusted to pH 10 with 2 N KOH. The free amine was extracted into ether, and the aqueous solution was adjusted to pH 7 with 0.1 N HCl. The solution was diluted to a final concentration of 0.2 μmole of mevalonate/0.1 ml. The number of counts per 0.1 ml was 5.4×10^4 cpm. Since only the L-isomer of the mevalonate forms cholesterol, the effective concentration is 0.1 μmole/0.1 ml containing 2.7×10^4 cpm. One-tenth milliliter of this solution was routinely added to each sample of liver homogenate.

A solution was prepared consisting of 22.5 g of KOH and 20 mg of cholesterol dissolved in 100 ml of 90% ethanol. This was used for the hydrolysis of cholesterol esters and for subsequent isolation of the total cholesterol. The phosphate buffer used was that of Bucher (11) with sucrose omitted.

Preparation of Biological Material. The general procedures of Bucher and McGarrahan (12) were followed. Sprague-Dawley rats were killed by cervical dislocation, their livers perfused *in situ* with cold buffer, excised, and homogenized. The homogenates were centrifuged at 0° for 10 minutes at 600 × *g*. The supernatant was used for incubations.

Each incubation mixture contained 2 ml of homogenate, to which was added 1 mg of ATP, 2 mg of DPN, 0.1 μmole of 1-mevalonate-2-C^{14}, and specified concentrations of inhibitory compounds. The homogenate with added cofactors was allowed to come to 38° in a Dubnoff shaker. Substrate and inhibitors were then added, the samples gassed with 95% O_2-N_2 for 1 minute, and then incubated for specified times.

Isolation of Cholesterol. The reaction was terminated by pouring the sample into an 18 mm × 150 mm Pyrex test tube containing 4 ml of KOH reagent. Cholesterol was isolated according to a procedure of Frantz and Bucher (13).

Each sample was heated overnight in a sealed tube at 85° in order to saponify esterified sterol. Cholesterol was removed from the hydrolysis mixture by three extractions into 10 ml of ether. The extracts were evaporated in 40-ml centrifuge tubes under a stream of nitrogen at 60° and the residues were triturated in a bath at 60° with 2 ml of alcohol-acetone 1/1 (v/v). The tubes were centrifuged, and the whole supernatant was transferred to 12-ml centrifuge tubes. To each sample 1 ml of 0.5% digitonin in 50% ethanol solution was added, and the mixture was chilled overnight. The resulting digitonide was plated on 2.4-cm circles of Whatman No. 42 filter paper and then washed with cold 50% ethanol solution. Each sample was dried, weighed, and then counted with a model M-5 Nuclear Chicago gas flow counter. All counting data were corrected to "infinite thinness."

Synthesis of Analogues of Mevalonic Acid. Figure 1 outlines the synthesis of the analogues of mevalonic acid. The general procedures of Kon and Linstead (14) were followed.

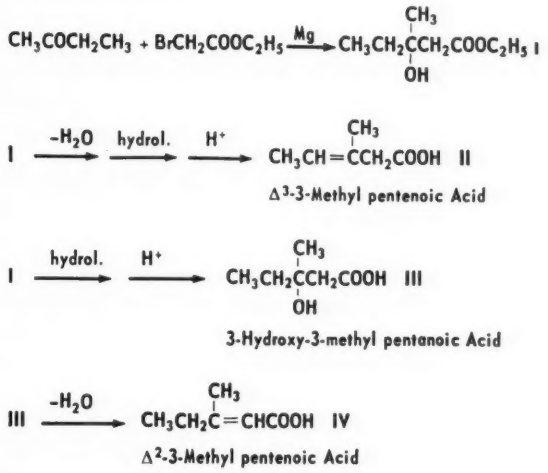

FIG. 1. Outline of synthesis of analogues of mevalonic acid. Compounds *II*, *III*, and *IV* were tested *in vitro*.

A Reformatsky reaction between methyl-ethyl ketone and ethyl bromoacetate was performed in the presence of magnesium with benzene as solvent. The resulting hydroxy ester was dehydrated by heating in the presence of $KHSO_4$. This procedure gave the unsaturated ester, which in turn was hydrolyzed in base to give the Δ^3 acid. Some hydroxy ester was hydrolyzed in alcoholic KOH to give the hydroxy acid. The latter compound was converted to the Δ^2 acid by heating it in the presence of acetic anhydride.

Analytical values and physical constants for the analogues are given below:

Δ^3-3-Methyl pentenoic acid

Anal. Calcd. for $C_6H_{10}O_2$: C, 63.11; H, 8.84. Found: C, 62.87; H, 8.66.

Constants: b.p., 55°–56.5° (0.03 mm Hg). n_D^{20}, 1.4471 (reported 1.4469 in Ref. 14). R_f, 0.71, in the chromatographic system (15): *n*-amyl alcohol-water-formic acid, 20:12:1, descending on Whatman No. 1 paper.

Δ^2-3-Methyl pentenoic acid

Anal. Calcd. for $C_6H_{10}O_2$: C, 63.11; H, 8.84. Found: C, 62.90; H, 9.02.

Constants: b.p., 67°–69° (0.03 mm Hg) m.p., 46°–48° (reported 48°–49° for the *trans* acid in Ref. 14). R_f, 0.63, in the *n*-amyl alcohol system.

Δ^3-Hydroxy-3-methyl pentonic acid

Anal. Calcd. for $C_6H_{12}O_3$: C, 57.62; H, 9.16. Found: C, 53.46; H, 8.93.

Constants: b.p., 93°–95° (0.03 mm Hg). n_D^{20}, 1.4442. R_f, 0.41, in the *n*-amyl alcohol system.

RESULTS AND DISCUSSION

Standard Incubation Conditions. Figure 2 illustrates the rate of incorporation of mevalonate-2-C^{14} into cholesterol under standard incubation conditions. The rate of sterol synthesis was linear for 10 minutes, and the system incorporated no more C^{14} into sterol after 15 minutes. A 5-minute incubation period was chosen for activity measurements since the curve was linear and about 5% conversion of mevalonic acid to cholesterol occurred at this time. Thus the effects of the inhibitory compounds were tested during a period of maximal rate of steroid synthesis.

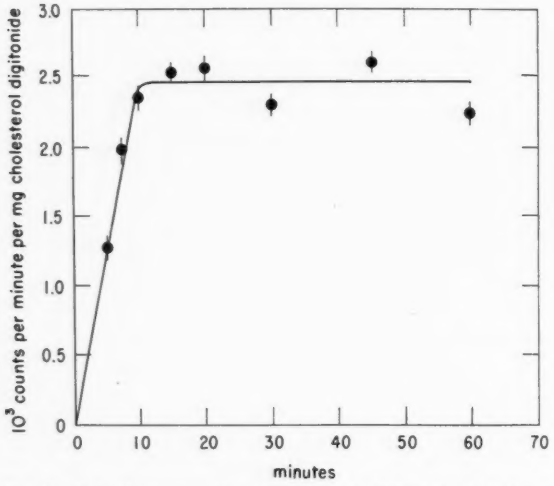

FIG. 2. Rate of incorporation of mevalonate-2-C^{14} into cholesterol in rat liver homogenates. Each point is the average of closely agreeing results of three samples.

Our choice of a 5-minute incubation time requires further comment. Although the reaction stops after 15 minutes in the absence of an inhibitor, it is possible that in the presence of an inhibitor the system might incorporate more C^{14} beyond the 15-minute period. This could occur if the activated mevalonic acid were metabolized solely by way of sterol biosynthesis. This appears to be the case from the work of Tavormina and Gibbs (6), Popják *et al.* (16), and Elwood *et al.* (17). If the inhibitor itself were metabolized, the duration of C^{14} incorporation into sterol could also be extended beyond 15 minutes since, as inhibitor concentration decreased, activated mevalonate would be converted to sterol. Therefore, if the incubation time had been the 2-hour period recommended by Frantz and Bucher (13) and Tavormina and Gibbs (6), the inhibition might have been less than during the 5-minute period of maximal rate of cholesterol synthesis. We have found in preliminary experiments that concentrations of inhibitors which produced 80% inhibition during a 5-minute incubation period caused a 65% to 70% inhibition during a 1-hour incubation time. This difference is probably significant since the counting error in the isotopic assay procedure did not exceed 5%.

Comparison of the Effects of the Three Compounds upon Cholesterol Biosynthesis. Figure 3 shows the effect of the three compounds in reducing the incorporation of isotope from mevalonate-2-C^{14} into cholesterol. The hydroxy acid is the most effective; the Δ^2 acid is next, and the Δ^3 acid is the least effective.

Half-maximal inhibition by the hydroxy acid occurred at a concentration of 5 μmoles/ml of homogenate. The ratio in μmoles of inhibitor to substrate is 100:1. The inhibition is probably not a result of changes in ionic strength since Bucher (11) found that the addition of 100 μmoles of α-ketoglutarate to samples of homogenate had no effect upon the incorporation of isotopic acetate into cholesterol. In the present system the addition of 5 μmoles of compound to 1 ml of homogenate would increase the molar concentration of the buffer by 10% at most.

Comparison of the Effects of Inhibitors in Tissue from the Same Animal. The data described so far were ob-

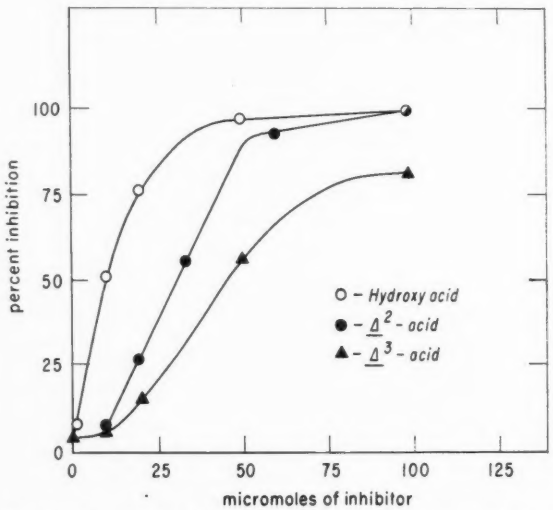

FIG. 3. Comparison of inhibitors. Each of the three acids was tested for its effect upon incorporation of mevalonate-2-C^{14} into cholesterol. Each point is the average of closely agreeing results from three samples.

tained by use of tissues from separate animals for each compound. In Figure 4 are compared the inhibitory effects of the three compounds at two different concentrations in tissue from the same animal. The results are essentially similar to those shown in Figure 3.

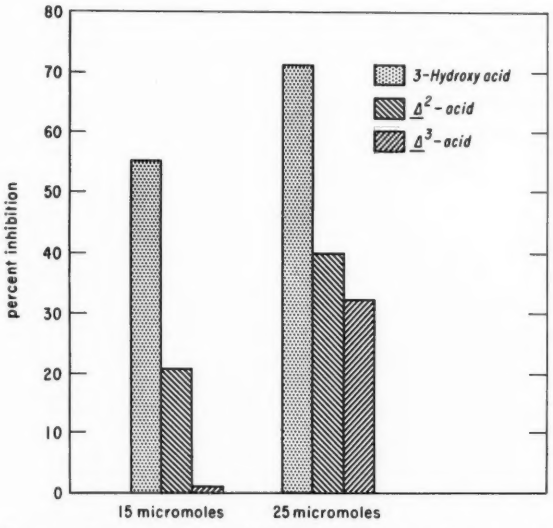

FIG. 4. Comparison of inhibitors in the same homogenate. Aliquots of the same homogenate containing C^{14}-mevalonate were incubated for 5 minutes with two different concentrations of each inhibitor. The height of each bar is the average of closely agreeing results from three samples.

As was mentioned above, the Δ^2 acid is more effective than the Δ^3 acid. This differs from the *in vivo* results of Mentzer *et al.* (3), who reported a converse relationship.

The Hydroxy Acid as a True Inhibitor. It is possible that the hydroxy acid might be converted to mevalonate in the homogenate. The reduced incorporation of isotope in the presence of the acid then could be explained by an increased pool of unlabeled mevalonate. If such a dilution is significant in the present system, it may be possible to decrease further the amount of isotope incorporation into sterol by preincubation of the inhibitor with homogenate before adding the labeled substrate.

To test this possibility, we preincubated hydroxy acid with homogenate. In the results described in Table 1, sample 1 is a control for sample 2, with inhibitor and substrate incubated together starting at zero time. Sample 3 is a control for samples 4 and 5. The latter samples were incubated with inhibitor for 15 and 5 minutes, respectively, before addition of substrate. Cofactors were added twice to these samples: once, together with the hydroxy acid in case any metabolism of this compound depended upon these cofactors;

TABLE 1. RESULTS OF PREINCUBATION STUDIES WITH INHIBITOR

Sample	C^{14}-Mevalonate at 0 Minutes	Cofactors at 0 Minutes			Hydroxy Acid at 0 Minutes			Sterol	Inhibition
		0	5	15	0	5	15	*cpm/mg*	*per cent*
1	+	+	−	−	−	−	−	1344	−
2	+	+	−	−	+	−	−	556	59
3	+	+	−	+	−	−	−	1869	−
4	+	+	+	−	−	+	−	894	52
5	+	+	−	+	−	−	+	909	52

The effect of preincubating homogenate with hydroxy acid is compared with the result of incubating samples to which C^{14} substrate and hydroxy acid were added at zero time. Each value is the average of three closely agreeing results.

for the second time, together with substrate in order to take into account the effects of adenosine triphosphatase and diphosphopyridine nucleotidase during the periods of preincubation.

The results indicate that there is no significant difference in the incorporation of isotope between the homogenates with which hydroxy acid has been preincubated and homogenates with which both acid and isotopic substrate has been incubated simultaneously.

Although these results are not conclusive, it appears that the hydroxy acid is stable for 20 minutes and acts as an inhibitor, not a diluent.

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The metabolism of palmitic acid-1-C^{14} in functionally hepatectomized rats*

BENGT BORGSTRÖM and THOMAS OLIVECRONA

Department of Physiological Chemistry,
University of Lund,
Lund, Sweden

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SUMMARY

Normal carbohydrate-fed rats and rats on which a functional hepatectomy had been performed by ligating the vessels of the porta hepatis were injected with C^{14}-labeled palmitic acid bound to albumin, and the distribution of activity studied. The concentration of free fatty acids (FFA) in plasma rapidly rises after the hepatectomy. The half life of the injected activity, however, does not seem to change significantly in these animals. In the normal rat a considerable fraction of the injected FFA is recirculated in the blood in glycerides and to a lesser extent in phospholipids. This recirculation is almost completely abolished after hepatectomy, indicating that the liver is the chief organ for plasma lipoprotein synthesis. The peripheral tissues are capable of esterifying the FFA but do not release any esterified fatty acids into plasma, with the possible exception of adipose tissue. There is less activity going into the adipose tissue after hepatectomy. The uptake of fatty acids into adipose tissue is discussed.

During recent years much interest has been focused on the metabolism of albumin-bound "free fatty acids" (FFA) in plasma (1, 2). They have a rapid turnover and seem to be the transport form of fatty acids mobilized from the fat depots (3). The flux of FFA from plasma, however, is considerably greater than the utilization of these acids for energy production (4). An equivalent flux of fatty acids back into adipose tissue is necessary if no net redistribution of fatty acids from adipose tissue to other tissues is to take place. Adipose tissue has been shown to take up only small amounts of the plasma FFA (5). A possible vehicle for transport of fatty acids back into adipose tissue might be lipoproteins synthesized by the liver. It is now well known that the liver takes up albumin-bound fatty acids (6) and gives them off as glycerides and phospholipids incorporated in plasma lipoprotein (7).

The purpose of the present study was to investigate the effect of functional hepatectomy on the recycling, as esterified fatty acid, of injected palmitic acid-1-C^{14} bound to albumin in plasma, and on the tissue distribution of the injected activity.

* This investigation has been supported by grants from the Swedish Medical Research Council and the Research Council of the Swedish Dairies Association.

EXPERIMENTAL

Fifty microcuries of palmitic acid-1-C^{14} (The Radiochemical Centre, Amersham, England; specific activity 28 mc/mmole) was dissolved in 1 ml ethanol, a moderate excess of NaOH was added, and the solution taken to dryness. Rat serum (5 ml) was added, the mixture stirred, and allowed to stand at room temperature for 1 hour before it was filtered. The filtrate was then stored deepfrozen until used. On analysis, more than 99% of the radioactivity was found in the form of FFA.

The experimental animals were male Sprague-Dawley rats, weighing 185 to 210 g at the time of injection. They were offered 10% glucose in 0.45% NaCl 12 to 20 hours before injection. No other food was given during this time. The functional hepatectomy was performed as follows: The rats were anesthetized with ether and the abdomen opened with a mid-line incision. The superior mesenteric artery was ligated to avoid engorgement of the splanchnic vessels with blood. Three minutes later the vessels entering and leaving the liver through the porta hepatis were ligated. Five minutes later 0.5 ml of palmitic acid-1-C^{14} serum mixture was injected into the left jugular vein and the rats were then kept anesthetized until sacrificed. Normal rats were injected in the same way but were not operated.

At the time of sacrifice as much blood as possible was drawn from the abdominal aorta and immediately centrifuged. An aliquot of serum was measured and poured into 20 volumes of chloroform-methanol 2/1 (v/v). The liver, the heart, and a piece of the left epididymal adipose tissue were cut up in approximately 20 ml of chloroform-methanol 2/1 (v/v) per g of tissue. The tissues were then homogenized in a Waring Blendor and allowed to stand overnight. The chloroform-methanol extracts were filtered and washed once with 0.4 volume 2% KH_2PO_4 and taken to dryness.

The extracts were separated on silicic-acid columns using a slight modification of Borgström's method (8, 9). The serum lipids were fractionated into a cholesterol ester fraction, a fraction containing glycerides and FFA, and a phospholipid fraction. The lipids of the other organs were separated into one fraction containing cholesterol esters, glycerides, and FFA, and one containing the phospholipids. The FFA were extracted from the neutral fat by dissolving the lipids in petroleum ether and washing twice with equal volumes of 0.1 N KOH in 50% ethanol. The ethanol phases were then acidified and extracted twice with equal volumes of petroleum ether. The petroleum ether was then evaporated and FFA determined by dissolving the residue in absolute ethanol and titrating. The rest of the neutral fat fraction in the first petroleum ether was hydrolyzed by adding an equal volume of 4% KOH in absolute ethanol. After the acidification, the fatty acids were extracted by adding equal volumes of water and petroleum ether. The lower phase was extracted once more with petroleum ether, the petroleum ether phases were taken to dryness, the residue dissolved in absolute ethanol and titrated. This extract contains the nonphospholipid esterified fatty acids and is called "neutral fat" in this article.

To the methanol eluate from the silicic-acid column an equal volume of 4 N KOH in water was added and the mixture refluxed for 4 hours. After acidification, the phospholipid acids were then extracted twice with equal volumes of petroleum ether. The petroleum ether was evaporated, and the residue was weighed, or dissolved in absolute ethanol and titrated. Finally, all fractions were acidified again, extracted, taken to dryness, dissolved in toluol containing 3% 2,5-diphenyloxazole (DPO), and counted in a liquid scintillation counter. By the use of a standard and an internal standard, all activities were subsequently corrected to an injected dose of 75,000 cps and a body weight of 200 g. The specific activity of the FFA of plasma after complete mixing was approximately calculated to 10,000 cps/μeq in the normal rats, and 3,400 cps/μeq in the hepatectomized rats. Each point on the curves represents results from one animal, but similar figures have been obtained in other series of experiments.

An additional series of experiments was undertaken to study more accurately the half time of the injected isotope. In these experiments three normal, three sham-operated, and three hepatectomized rats were injected as above, and 0.5 ml of blood taken from the jugular vein 1, 2, 4, 6, 8, 12, and 16 minutes after the injection. Plasma (0.2 ml) was extracted and the total activity determined.

RESULTS

Disappearance of Injected Material. The concentration of FFA in plasma is 0.62 ± 0.12 μeq/ml in the normal rats and 2.76 ± 0.92 μeq/ml in the hepatectomized rats. The half life of the injected activity is 0.8 minute in the normal rats and 1.0 minute in the hepatectomized (Fig. 1). These half-life figures are approximate as they are calculated on the estimated zero-time dilution of injected label and the observed values after 5 minutes. In these calculations it has also been assumed that the blood volume is not changed after hepatectomy. In the experiments in which blood from the same animals was frequently sampled, half-life figures between 2 and 4 minutes were 1.6, 1.7, and 1.7 minutes and between 4 and 6 minutes 3.2, 3.4, and

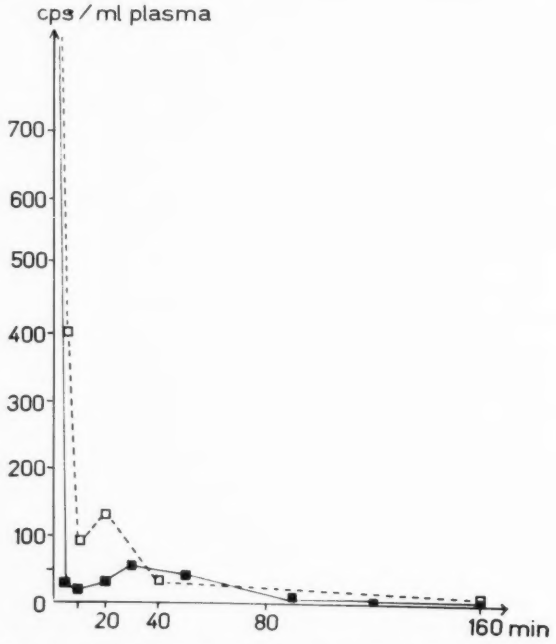

FIG. 1. Disappearance of injected albumin-bound palmitic acid-1-C^{14} from rat plasma. Solid lines: normal rats. Broken lines: hepatectomized rats.

3.2 minutes for the normal, sham-operated, and hepatectomized rats, respectively. Calculating the zero-time dilution of injected label from a plasma volume of 12 ml in a 200-g rat gave half lives for the three types of rats during the first 2 minutes of 0.9, 0.8, and 1.4 minutes. If the half life during this time in the hepatectomized rats is assumed to be the same in the normal, their plasma volume can be calculated to about 8 ml.

Labeling of Other Plasma Lipids. In the normal rats the plasma neutral fat fraction soon becomes labeled (Fig. 2). After about 15 minutes its specific activity is higher than that of the FFA in the plasma, and it stays higher during the rest of the observed time. The phospholipids are labeled more slowly. Their maximum specific activity is 27 and is reached after 50 minutes; the maximum specific activity of the triglycerides is 300 and is reached 30 minutes after the injection, making up around 3.5% of the specific activity of the FFA fraction of plasma as calculated at zero time.

In the hepatectomized rats, on the other hand, no significant labeling of the plasma triglycerides or phospholipids can be shown. The low activities shown after 5 minutes can well be ascribed to contamination from the FFA fraction during the analysis.

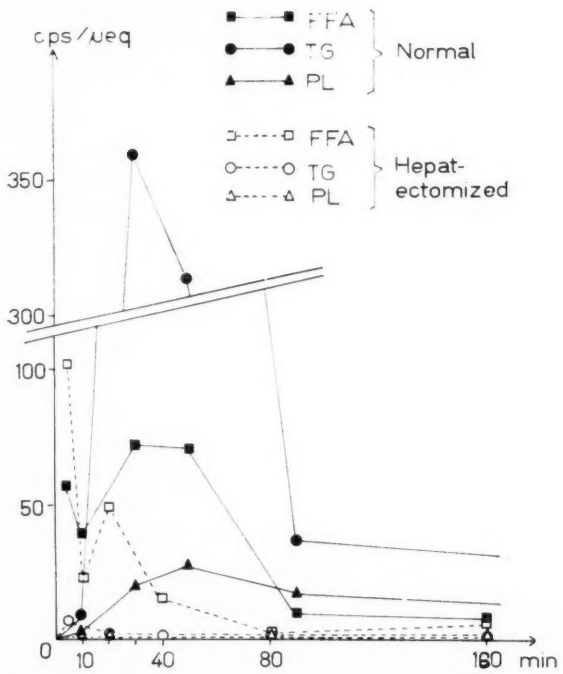

FIG. 2. Specific activity of the fatty acids in the plasma lipid fractions after the injection of albumin-bound palmitic acid-1-C^{14}.

Labeling of the Liver Lipids. The liver lipids are labeled soon after the injection of labeled FFA (Fig. 3). The triglycerides reach their maximum specific activity 10 minutes after the injection, and then fall off at a rather constant rate during the next 70 minutes. The phospholipids reach their maximum specific activity 90 minutes after the injection. At this time the lipid fractions seem to have equilibrated, and their specific activities then fall off at about the same rate. In the hepatectomized rats the livers were cut out after sacrifice, and were shown to contain less than 0.5% of the injected activity.

Labeling of the Heart Lipids. The heart was chosen as a typical peripheral tissue using FFA for energy production. It is obvious from Figure 4 that a large proportion of the label is contained in the neutral fat fraction, both in the normal and in the hepatectomized rats. This indicates that the fatty acids are esterified in the heart itself and not transported there from the liver, as the plasma in the hepatectomized rats was shown to contain almost no labeled esterified fatty acids.

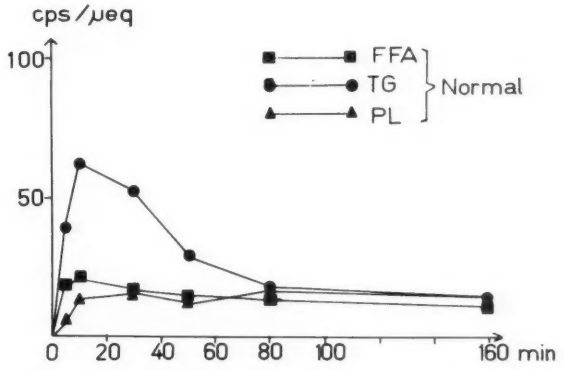

FIG. 3. Specific activity of the fatty acids in the liver lipid fractions after the injection of albumin-bound palmitic acid-1-C^{14}.

Labeling of the Adipose Tissue Lipids. The hepatectomized rats have more FFA in their depots than the normal ones: 0.5% to 2.0% of the total amount of fatty acids of the depots are free in the hepatectomized rats compared with 0.1% to 0.3% in the normal ones. Figure 5 shows the total amount of activity present in 1 g of epididymal adipose tissue in the different fat fractions rather than the specific activity. There is less activity going into the adipose tissue of the hepatectomized rats, which might well be due to the fact that the direction of fatty acid transport in these animals is largely out of the adipose tissue.

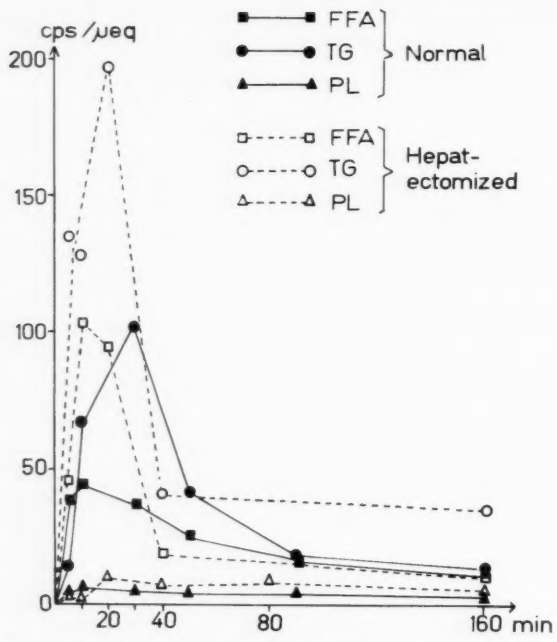

FIG. 4. Specific activity of the fatty acids in the heart lipid fractions after the injection of albumin-bound palmitic acid-1-C^{14}.

DISCUSSION

The increased concentration of plasma FFA in hepatectomized animals can be the result of an increased mobilization of fatty acids from the fat depots, or a decreased utilization of fatty acids, or a combination of both. The increase in the FFA pool of adipose tissue found in these animals might indicate an increased outflow of fatty acids from this tissue. A decrease in the plasma volume after hepatectomy also contributes to this increase of FFA concentration.

The most interesting conclusion to be drawn from this work is that the liver is necessary for the incorporation of FFA of plasma into plasma lipoprotein glycerides. Although other organs, such as the heart, synthesize triglycerides from the FFA fraction of plasma, they are not given off to the circulation. The possibility exists that the liver is the only organ, beside the small intestinal mucosa, which can synthesize lipoproteins of plasma type. That the same holds true for phospholipid has been shown by Fishler *et al.* (10). The possibility that the adipose tissue is liberating triglycerides into the plasma cannot, however, be ruled out, as the specific activity of the adipose tissue triglycerides remains very low during the observation time. The importance of the liver for the synthesis of plasma glycerides has also been stressed recently by Byers and Friedman (11), who found that hepatectomy abolished triglyceridemia after injection of Triton WR-1338 into rats.

A common feature for the liver and the heart is that the specific activity of the triglyceride fatty acids soon rises above that of the FFA from which they probably are derived. This fact may be explained in light of the findings of Stein and Shapiro (7), that within the liver cells there is a much higher specific activity in the particle-bound triglycerides than in those of the

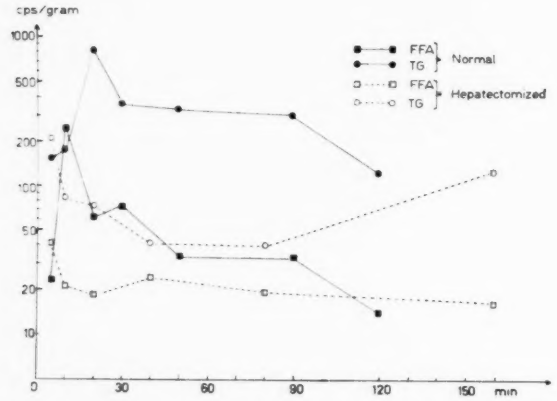

FIG. 5. Total activities of the fatty acids in the epididymal adipose tissue lipid fractions after the injection of albumin-bound palmitic acid-1-C^{14}. The figures are given per gram of wet tissue weight.

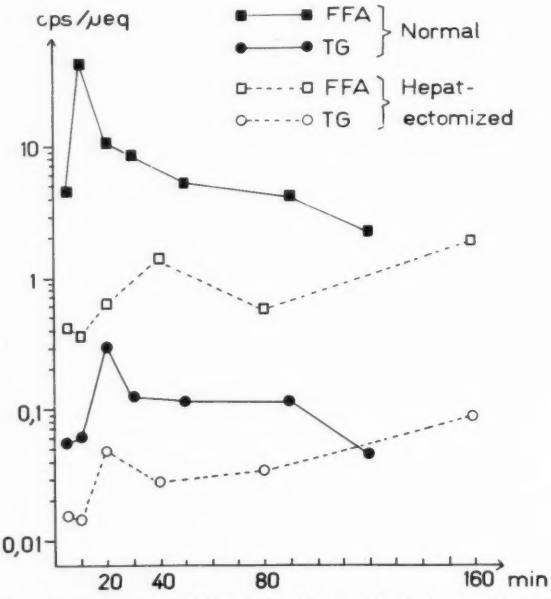

FIG. 6. Specific activities of the fatty acids in the epididymal adipose tissue lipid fractions after the injection of albumin-bound palmitic acid-1-C^{14}.

floating fat. The same is in all probability true for lipid fractions of other organs. This means that the FFA coming into the cells from the plasma and having high specific activity is directed preferentially to the actively metabolizing sites in the cells, that is, the particles, where they are built into different forms of fatty acid esters. One must also assume that in the liver these newly synthesized triglycerides and phosphatides having higher specific activity than the bulk of the liver fat are preferentially built into lipoproteins. This may explain the fact that the plasma triglycerides have a higher specific activity than the total liver triglycerides.

In the adipose tissue of the normal rats the FFA fraction reaches its maximum activity 10 minutes after the injection of the labeled palmitic acid (Fig. 6). It then gradually falls off during the observation time. The triglycerides are rapidly labeled and reach their maximum specific activity 20 minutes after the injection. The activity then falls off during the first 2 hours. Other experiments carried out in this laboratory have shown that there is a secondary rise of activity in the adipose tissue glycerides in carbohydrate-fed rats. This starts after about 2 hours and continues for at least 24 hours.[1] This can be interpreted to mean that the FFA coming into the adipose tissue are directed to the "actively metabolizing sites," where they are rapidly built into esters. The main part of these esters is almost immediately broken down again, and the fatty acids mobilized from the adipose tissue. This means that a rather rapid exchange of fatty acids between plasma and adipose tissue takes place, but that only a small fraction of the adipose tissue fatty acids is readily exchangeable with those of plasma. However, a small fraction of the newly synthesized esters leaks out into the floating fat fraction and remains there, being turned over at a much lower rate than the fat on the "actively metabolizing centers." This fraction accounts for the secondary slow rise of activity.

In the hepatectomized rats the same trend can be seen. However, both the FFA and the triglycerides reach their maximum specific activity faster than in the normal rats, and the activity going into adipose tissue is lower. As was mentioned before, these rats seem to be mobilizing fatty acids from their depots at a rapid rate. This means that the rate of exchange between plasma and adipose tissue is higher than in the normal rats and that the direction of fatty acid transport is mainly out of adipose tissue. If this is so, it could well explain the fact that the activity rises and falls off faster and stays at a lower level in these rats than in the normal ones. Another explanation that cannot be ruled out is that the adipose tissue normally takes up preferentially plasma esterified fatty acids. Bragdon and Gordon (5) have shown that more activity goes into the adipose tissue after the injection of fatty acids incorporated into chylomicrons than after injection of FFA. In the normal rats in the present study a large fraction of the injected activity is recycled in the plasma as esterified fatty acids, while in the hepatectomized rats no such recycling takes place. If the adipose tissue is specialized on the uptake of esterified fatty acids, it is obvious that less activity should appear in the adipose tissue in the hepatectomized rats.

[1] Thomas Olivecrona, unpublished observation.

The skillful technical assistance of Miss Iréne Lindell is gratefully acknowledged.

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The free fatty acids bound to human serum albumin*

ABRAHAM SAIFER and LAWRENCE GOLDMAN

Department of Physical Chemistry, Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn 3, New York

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SUMMARY

The fatty acid composition of human serum albumin (Cohn Fraction V) was determined by gas-liquid chromatography. Forty-three peaks were observed; 26 of these were identified by various methods. Eight of the identified peaks constituted about 90% of the total free fatty acids, and 65% of the total were unsaturated acids. The patterns of fatty acids bound to albumin were essentially the same as those found in plasma. However, quantitative differences were observed in the percentage of 18 carbon acids in the free fatty acids of human serum albumin samples as compared to plasma. Possible explanations to account for these differences are discussed.

The free fatty acids (FFA) present in human blood plasma represent a transport form of lipid readily available as substrate for energy production (1). FFA exist in plasma primarily as anions and are "free" only in the sense of not being bound by covalent linkages. Less than 0.01% are actually free in solution. Although a small percentage of FFA is normally bound to lipoproteins, the entity of greatest biological significance is the albumin-fatty acid complex (2). One of the main physiological roles of the serum albumins is to serve as a transport vehicle for many kinds of anions, including FFA. In previous publications from this laboratory, the anomalous behavior of human serum albumin in the Tiselius electrophoresis apparatus (3), and the effect of bound lipids on the electrophoretic patterns, have been investigated at acid pH (4). Human serum albumin prepared by low temperature-ethanol fractionation (Cohn Fraction V) gives a single electrophoretic boundary in barbital buffer, pH 8.6, 0.10 ionic strength (μ), but multiple boundaries in the acid pH region. Removal of lipid from the albumin preparation by ether (5), or isooctane (6) treatment at low temperature, yields samples which give a single ascending boundary in both 0.10 μ acetate (pH 4.0) and barbital (pH 8.6) buffer systems.

This paper deals with the composition of the fatty acids removed from human serum albumin by denaturation of the protein and by low temperature-ether treatment of albumin solutions. Results obtained for FFA bound to human serum albumin were compared with those found by Dole *et al.* (7) for human plasma.

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EXPERIMENTAL

Isolation of Free Fatty Acids from Albumin. The human serum albumin employed in this procedure was supplied in the form of a 25% solution (Squibb Fraction V) containing 0.04 M acetyl tryptophanate as a stabilizer.[1] The absence of cholesterol and its esters, triglycerides, and lipid phosphates was verified by silicic-acid chromatography (8). For the gas-liquid chromatographic study of the FFA bound to albumin, the protein was subjected to the denaturation-extraction procedure of Cohn *et al.* (9). This was necessary because of the small amount of bound lipid, which cannot be completely removed without denaturing the protein (10). Solvent was removed from the extract by low temperature vacuum distillation and the residue extracted with petroleum ether.

Methylation and Gas-Liquid Chromatography of Esters. For gas-liquid chromatography, the acids were methylated directly on an anion exchange resin, IRA-400, as described by Hornstein *et al.* (11). The methyl esters were chromatographed at 197° on a nonpolar stationary phase, Apiezon L, and then on ethylene glycol adipate, a polar stationary phase. A Pye Argon

[1] This albumin was supplied gratis by James H. Pert, Research Director, American National Red Cross, Washington, D. C.

Chromatograph, employing the Sr 90 ionization chamber of Lovelock (12) as a sensing device, was used. Acids were identified by five methods: addition of known components, comparisons with the retention timetables published by the Rockefeller Institute group (13), peak reversal on Apiezon and ethylene glycol adipate columns, the linear relationship between the logarithm of the retention time and the number of carbon atoms in a homologous series, and by microbromination. Quantitation was accomplished by measurement of the areas under the peaks with a planimeter.

RESULTS

Gas-liquid chromatography revealed the presence of at least 43 different acids, of which 26 were identified. Eight acids constituted about 90% of the total: oleic 33; palmitic 25; linoleic 20; arachidonic 5; palmitoleic 3; and myristic, stearic, and 20:3, each about 1.5% of the total. Approximately 66% of the total fatty acids were unsaturated, about 1% were odd-numbered, and less than 1% C_{10} or shorter. Branched-chain and polyunsaturated fatty acids appeared to be present in trace amounts. Table 1 represents the percentage by weight of the fatty acids of albumin found in all samples tested, in comparison with those reported by Dole *et al.* (7) for human blood plasma. The "double bond index" (7) shown in Table 1 is a measure of unsaturation, calculated as $\Sigma(n_i w_i/m_i)$, where n_i, is the number of double bonds in the fatty acid "i," m_i is the molecular weight, and w_i the per cent by weight of the fatty acid in the given material.

DISCUSSION

The compositions of the FFA removed by denaturation of albumin shown in Table 1 are similar to those reported by Dole *et al.* (7) for the FFA of human plasma. The differences obtained reside in the per cent composition of 18:0, and in the degree of unsaturation. The nature of the materials which were methylated may in part account for the differences noted in the composition of the FFA in human serum albumin, as compared to human plasma. Trout *et al.* (14) have suggested that some phospholipid is probably extracted and titrated in the Dole method (15). If this is so, gas-liquid chromatography of the methyl esters recovered by Dole's method may represent not only those acids bound to albumin, but also the fatty acids constituting part of the plasma phospholipid fraction as well.

Gas-liquid chromatography of the methylated FFA, removed by low temperature-ether treatment of albumin solution, showed patterns similar to those obtained by denaturation of the protein. An essential difference involved the 16:0 and 18:0 components which were present to about the same extent, i.e., 29% in the case of the ether-treated albumin. This is not unexpected, since extraction at low temperature does not remove all the lipid (or FFA) from albumin. Oleate, exhibiting the greatest affinity for albumin (2), would be more completely removed from albumin only after denaturation.

The relatively stronger binding affinity of unsaturated FFA, such as oleate, to albumin, as compared to saturated acids, e.g., stearate, as found by Goodman (2), has been confirmed by means of moving boundary electrophoresis at acid pH (4). Experimental work now in progress employs a combination of electrophoretic and gas-liquid chromatographic procedures for

TABLE 1. THE FREE FATTY ACID COMPOSITION OF HUMAN SERUM ALBUMIN (FRACTION V) IN COMPARISON WITH THOSE PRESENT IN HUMAN BLOOD PLASMA

Free Fatty Acids* in Albumin		Free Fatty Acids in Plasma†
	per cent	*per cent*
Saturated		
12:0	<1	<1
13:0	<1	
14:0	1.5	1.7
15:0	<1	
16:0	24.7	23.2
17:0	<1	
18:0	1.5	12.9
20:0	<1	
Monounsaturated		
14:1	<1	<1
16:1	3.1	2.4
18:1	33.1	28.9
18:1‡		3.7
Diunsaturated		
18:2	20.0	14.5
Polyunsaturated		
18:3	<1	
20:3	1.4	1.3
20:4	5.0	4.7
20:5	<1	
Double bond index	36.0	28.0

* The system of nomenclature used in this table is the same as that listed in reference 13.

† Values given are those reported by Dole (7).

‡ Isomers.

determining the binding affinities of various FFA to albumin.

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Separation of dog serum lipoproteins by ultracentrifugation, dextran sulfate precipitation, and paper electrophoresis*

Toshio Sakagami† and D. B. Zilversmit‡

Division of Physiology,
University of Tennessee,
Memphis 3, Tennessee

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SUMMARY

The separation of dog serum lipoproteins by ultracentrifugation at a density of 1.063 is hindered by the failure of the high density fraction to accumulate in the bottom portion of the centrifuge tube. This phenomenon interferes with the quantitative recovery of pure low density lipoproteins. By paper electrophoresis, a lipid-containing protein with the mobility of a β-globulin was detected in the lowest layer of the centrifuge tube. Comparison of the preparative ultracentrifuge technique with that of dextran sulfate precipitation of β-lipoproteins revealed the suitability of the latter procedure for the quantitative separation of β-lipoproteins. The dextran sulfate β-lipoprotein precipitate was shown to be free from α-lipoprotein by paper electrophoresis.

The separation of dog serum lipoproteins into an α- and a β-, or a high density and low density fraction, is complicated by the fact that in dog serum the amount of β- compared to α-lipoprotein is very small. It is of interest, therefore, to determine which one of several available procedures gives the most complete and specific separation of the lipoprotein fractions. If the newer precipitation procedures utilizing dextran sulfate could be shown to yield quantitative results, they would be superior to ultracentrifugation because of their simplicity and rapidity of performance.

Precipitation of β-lipoproteins with sulfated polysaccharide has been studied by Burstein and Samaille (1), Castaigne and Amselem (2), Bernfeld *et al.* (3), Oncley *et al.* (4), and Florsheim and Gonzales (5). Moreover, turbidimetric procedures based on the precipitation of β-lipoproteins with various polysaccharides have also been developed by Antoniades *et al.* (6) and Boyle and Moore (7). Although the principle of precipitating β-lipoproteins with sulfated polysaccharides is the same in all these procedures, the details of the technique vary considerably. Some of the authors utilized calcium ions in addition to a relatively low molecular weight dextran sulfate (1, 2, 5), whereas others used higher molecular weight polysaccharide sulfates without calcium (3, 4). Boyle and Moore (7), on the other hand, utilized an agar preparation. We examined a modification of the Burstein procedure suggested by Castaigne and Amselem (2), since the dextran sulfate they used is available from commercial sources.

EXPERIMENTAL

Blood from normal fasting male dogs was obtained carefully to avoid hemolysis, and was allowed to clot. The precipitation and isolation of the β-lipoprotein with dextran sulfate[1] was carried out as follows: To each milliliter of serum was added 0.04 ml of 5% dextran sulfate and 0.1 ml of 11% calcium chloride. After standing 2 hours at 4°, the mixture was centrifuged for 10 minutes at about 4,000 × *g* and the supernatant removed by decantation. For subsequent paper electrophoresis and lipid analysis, the precipitate was redissolved in 0.5 ml of 0.9% sodium chloride, and calcium ions were removed by the addition of 0.05 ml of a solution of 12.7% potassium oxalate and centrifugation (2).

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† Postdoctoral Research Fellow from Sapporo Medical College, Japan.

‡ Career Investigator of the American Heart Association.

[1] Dextrarine, 2-ml ampoule containing 200 mg dextran sulfate. Obtained from l'Equilibre Biologique S. A., Commentry (Allier), France.

The ultracentrifugal separations were carried out in a Spinco Model L ultracentrifuge with a 40 rotor, essentially by the method of Havel *et al.* (8). In this procedure 10 ml of serum is adjusted to a density of 1.063 with NaCl–KBr solution, and centrifuged for 14 hours at 8° and an average acceleration of 105,000 × *g*. In later experiments the time of centrifugation was increased from 14 to 24 hours, and the amount of serum was decreased from 10 to 5 ml. To avoid the presence of radioactive potassium in preparations subsequently used for counting, sodium bromide was substituted for potassium bromide.

Paper electrophoresis was performed on a Spinco Model R unit with 10 to 50 lambda serum samples. In most instances electrophoresis was performed with a Spinco Model R unit, containing eight paper strips (S and S 2043 A g1), at room temperature for 16 hours with a current of 5 ma. Protein fractions, dissolved in concentrated salt solutions, exhibited electrophoretic mobilities lower than those obtained in whole serum. In one experiment we compared the mobility of crystalline bovine albumin dissolved in 0.9% or 5% sodium chloride In a parallel ultracentrifugation (d = 1.063) experiment seven layers obtained from the centrifuge tube were subjected to paper electrophoresis before and after dialysis against 0.9% sodium chloride containing 0.01% EDTA.[2] The mobilities of albumin and α-globulin in the higher salt concentration were decreased, even though only 50 lambda of the protein solutions were applied to the paper strip wetted with 0.075 M Na-barbital buffer pH 8.6. After dialysis the mobilities of these protein fractions were restored to normal.

At the termination of the electrophoresis, the strips were cut lengthwise into two portions: one portion was stained with bromophenol blue (9), and the other portion was stained with Sudan black B by the method of Moinat *et al.* (10). Lipids from serum and saline solutions after ultracentrifugation were extracted with 50 volumes of chloroform-methanol 2/1 (v/v) by an adaptation of the procedure of Folch *et al.* (11). After washing the chloroform-methanol extract with upper phase containing 0.02% $CaCl_2$, lipid phosphorus was estimated by a slightly modified method of Bartlett (12). The amount of phospholipid was obtained by multiplication of the phosphorus by 25. An aliquot of the extract was hydrolyzed with 2% KOH at 60° for 20 minutes, and extracted by low boiling petroleum ether (Skellysolve F, b.p. 35.5°–57°), according to the method of Abell *et al.* (13). The colorimetric determination of cholesterol was performed by the ferric chloride-sulfuric acid procedure of Zak *et al.* (14).

[2] EDTA = ethylenediamine-tetraacetate.

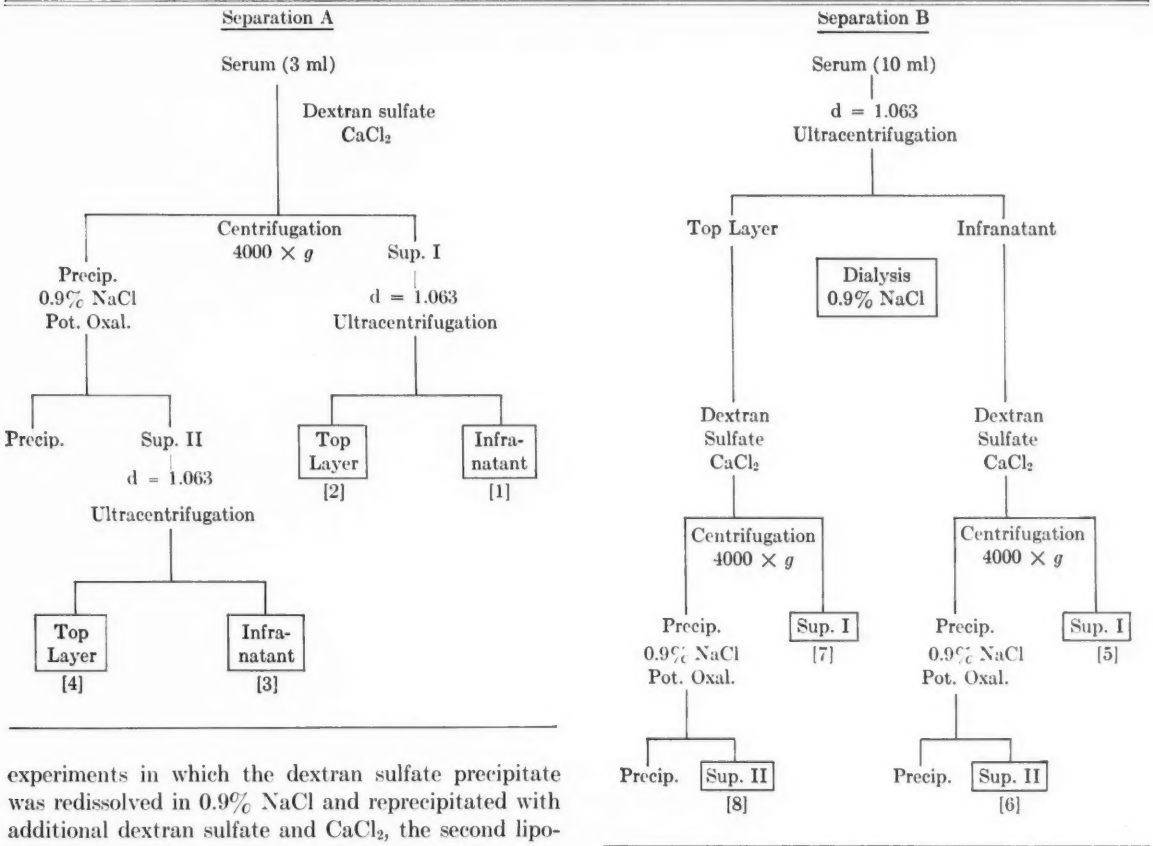
RESULTS

To compare the dextran sulfate precipitation with ultracentrifugation a single dog serum sample was divided into a 3- and a 10-ml portion. The 3-ml portion was precipitated first by dextran sulfate, as indicated by Table 1. After centrifugation the supernatant, Sup. I, was separated by ultracentrifugation (d = 1.063, 14 hours, 8°, 105,000 × *g*) into a high density lipoprotein, fraction [1], and a low density lipoprotein, fraction [2] by the use of a Spinco tube slicer. The dextran sulfate precipitate was redissolved in 0.9% NaCl, and Ca^{++} removed by the use of potassium oxalate. The saline solution, Sup. II, was ultracentrifuged to provide a high density lipoprotein [3] and a low density fraction [4].

The 10-ml serum aliquot was first subjected to ultracentrifugation at a density of 1.063 for 14 hours. Both the low density and the high density fractions, after being removed from the tube with the aid of a tube slicer, were dialyzed against 0.9% sodium chloride containing 0.01% EDTA for 24 hours. After adjusting the volumes to 25 ml, 5-ml aliquots were precipitated with dextran sulfate and calcium chloride, centrifuged, and precipitates were redissolved to furnish fractions [5], [6], [7], and [8] (Table 1). The results of cholesterol and phospholipid analyses of the 8 fractions listed in Table 1 are given in Table 2. In separation A, supernatant number I, obtained after dextran sulfate precipitation, should be α-lipoprotein. Accordingly, the high density lipoprotein fraction [1] in Table 2 contains more than 10 times the amount of cholesterol than fraction [2]. The small amount of cholesterol-containing material in fraction [2] might be either low density lipoprotein that had not been precipitated with dextran sulfate, or it might represent high density lipoprotein which had not centrifuged down after 14 hours at 105,000 × *g*. Apparently the latter is the case, since the cholesterol to phospholipid ratio of fraction [2] is very close to that for fraction [1] and is typical for the known cholesterol to phospholipid ratio of high density lipoprotein.

Fractions [3] and [4] are derived from the dextran sulfate precipitate and one would therefore expect the majority of the lipid to be in fraction [4], which represents the low density lipoprotein component. According to Table 2, more than 80% of the cholesterol is indeed present in fraction [4]. This lipoprotein has a cholesterol to phospholipid ratio of 0.84. The 20% of the cholesterol present in fraction [3], with a cholesterol to phospholipid ratio characteristic of the high density lipoprotein, actually represents a contamination of the dextran sulfate precipitate. In subsequent

TABLE 1. SEPARATION OF LIPOPROTEINS BY COMBINED DEXTRAN SULFATE PRECIPITATION AND ULTRACENTRIFUGATION



experiments in which the dextran sulfate precipitate was redissolved in 0.9% NaCl and reprecipitated with additional dextran sulfate and $CaCl_2$, the second lipoprotein precipitate was shown to be free of α-lipoprotein component as shown by paper electrophoresis (see Fig. 2, strip 4). Although the staining of lipoproteins with Sudan black B is not an extremely sensitive procedure, the application of a very concentrated β-lipoprotein solution to strip 4 and the absence of a visible alpha band would appear to eliminate appreciable contamination with α-lipoprotein.

In separation B, in which ultracentrifugation was carried out first, the low density and high density lipoprotein fractions had to be dialyzed, since dextran sulfate will not precipitate β-lipoproteins quantitatively in the presence of a high salt concentration. After dialysis, the high density lipoprotein fraction was precipitated with dextran sulfate. Very little cholesterol was found in the precipitate (fraction [6]), and the little lipoprotein that did precipitate exhibited a low cholesterol to phospholipid ratio, which indicates that it was high density lipoprotein.

The low density ultracentrifugal lipoprotein fraction was shown to contain an appreciable amount of high density fraction [7] that did not precipitate with dextran sulfate, and exhibited a low cholesterol to phospholipid ratio. This contamination is clearly the result of poor ultracentrifugal separation or cutting the centrifuge tube at too low a level.

In order to investigate the distribution of dog serum lipoproteins along the length of the centrifuge tube after ultracentrifugation, a 10-ml serum sample was centrifuged according to the method of Havel *et al.* (8).

TABLE 2. CHOLESTEROL AND PHOSPHOLIPID IN FRACTIONS OF TABLE 1

	Sup. I		Sup. II		HDL		LDL	
	[1] Infr.	[2] Top	[3] Infr.	[4] Top	[5] Sup. I	[6] Sup. II	[7] Sup. I	[8] Sup. II
Cholesterol	67.5	6.6	2.0	8.8	64	2.4	12.8	9
Phospholipid	185	16.6	5.9	10.4	150	5.6	29.5	10.5
Cholesterol/ phospholipid	0.36	0.40	0.34	0.85	0.43	0.43	0.43	0.86

All values are expressed as mg/100 ml of original serum.

TABLE 3. DISTRIBUTION OF DOG SERUM CHOLESTEROL AND PHOSPHOLIPID IN CENTRIFUGE TUBE

		10 ml Serum, 14 Hours Ultracentrifugation							Recovery
		Top Layer	Infranatant Fractions						
			1	2	3	4	5	6	
Cholesterol	*mg*	0.80	1.02	1.33	1.35	1.32	1.35	2.07	9.24
	*per cent**	9.1	11.5	15	15.1	14.7	15.1	23.2	103.9
Phospholipid	*mg*	1.35	1.89	2.75	3.36	2.80	3.23	4.72	20.1
	*per cent**	7	12.8	12.8	14.5	14.6	16.8	24.6	104.5
Cholesterol/ phospholipid		0.59	0.54	0.48	0.40	0.47	0.42	0.44	
		5 ml Serum, 24 Hours Ultracentrifugation							
Cholesterol	*mg*	0.50	0.21	0.34	0.48	0.77	1.10	0.98	4.38
	*per cent**	11.4	4.8	9.1	10.9	17.5	25	22.2	99.6
Phospholipid	*mg*	0.83	0.46	0.71	1.02	1.71	2.56	2.41	9.70
	*per cent**	8.6	4.8	7.4	10.6	17.8	26.6	25	100.5
Cholesterol/ phospholipid		0.60	0.45	0.48	0.47	0.44	0.43	0.40	

* Per cent of amount in original serum which contained: Cholesterol: 8.80 mg/10 ml; phospholipid: 19.2 mg/10 ml. Cholesterol/phospholipid ratio: 0.46.

Table 3 shows the concentration of cholesterol and phospholipid in various layers of the centrifuge tube after a 14-hour centrifugation at 105,000 × *g*. The "top layer," which contained about 1 ml of the uppermost layer in the centrifuge tube, was turbid and considered to contain predominantly low density lipoproteins. The rest of the centrifuge tube was divided as equally as possible into six divisions, containing approximately 2 ml each, numbered from 1 to 6, from the top to the bottom of the tube. It is apparent from the data in Table 3 that both cholesterol and phospholipids are distributed throughout the tube, although infranatant fraction 1 contains somewhat less lipids than the other fractions. Cholesterol to phospholipid ratios of the fractions near the top were higher than those in the bottom fractions. Apparently some contamination of the top layers with so-called high density lipoprotein existed. In Table 3 are also reported the results of a similar experiment, except that only 5 ml of the same serum was centrifuged, and the centrifugation was carried on for 24 hours. It is apparent that in the fractionation of 5 ml over a longer time period there is a better separation between the high density and low density layers. Infranatant fraction 1 contains only 4.8% of the total cholesterol, as compared to 11.5% in the previous experiment. Although Table 3 represents only two experiments, we have repeated these procedures each four times, and the results are consistent with one another.

The results of these analyses are confirmed by paper

TABLE 4. CHOLESTEROL PRECIPITATION AT VARIOUS TIMES AFTER ADDITION OF DEXTRAN SULFATE AND CALCIUM CHLORIDE TO 1 ML DOG SERUM

		10 Min	30 Min	60 Min	2 Hrs	5 Hrs	24 Hrs
Supernatant*	*mg*	0.837	0.827	0.784	0.779	0.801	0.797
	per cent‡	92.6	91.5	86.6	86	88.5	88
Precipitate†	*mg*	0.0787	0.0805	0.109	0.112	0.109	0.109
	per cent‡	0.6	08.9	12	12.4	12.1	12
Total recovery	*mg*	0.915	0.907	0.893	0.891	0.91	0.906
	per cent‡	101	100	98.6	98.4	101	100

* α-Lipoprotein.
† β-Lipoprotein.
‡ Per cent of amount in original serum which contained 0.905 mg cholesterol/ml.

electrophoresis. In both ultracentrifugal runs the top layers (Figs. 1*a* and 1*b*) contained β-lipoprotein. In the 10-ml sample a heavy alpha band was visible in infranatant layer 1 (Fig. 1*a*), and a little β-lipoprotein could be discerned. In the 5-ml sample neither alpha nor beta bands were visible in infranatant layers 1 and 2 (Fig. 1*b*). In lower layers of both experiments α-lipoprotein was the only band visible except in the very bottom layer (infranatant 6, Figs. 1*a* and 1*b*). Here a definite Sudan stain in the beta region was visible.

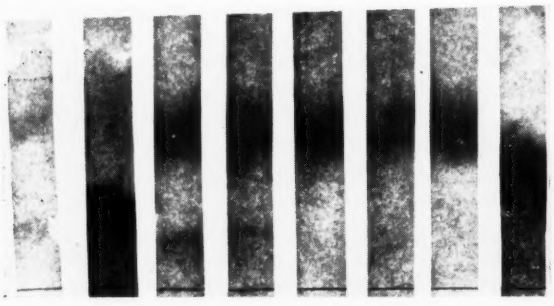


FIG. 1*a*. Paper electrophoretograms (Sudan black B stain) of whole serum (w.s.) and seven layers from top to bottom after ultracentrifugation of 10 ml of serum at density 1.063 for 14 hours in a 40 rotor. After dialysis all fractions were concentrated approximately threefold with the aid of Carbowax® (17).

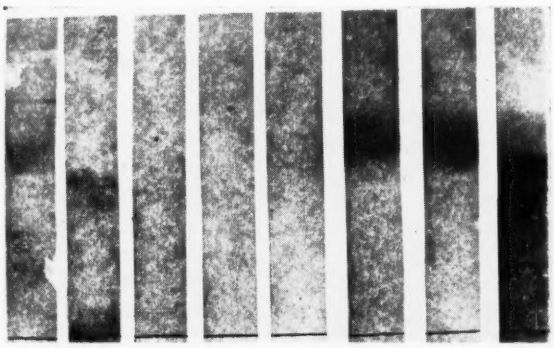


FIG. 1*b*. Same as Fig. 1*a*, except that 5 ml of serum was centrifuged for 24 hours. Photographs were made with transmitted light.

In order to circumscribe more precisely the conditions under which complete precipitation of β-lipoproteins in the presence of dextran sulfate and calcium occurs, six 1-ml serum samples from a fasting male dog were subjected to dextran sulfate precipitation, and the precipitate, as well as the supernatant, was analyzed for cholesterol after various periods of standing at 4°. Table 4 shows the results of one experiment with samples 10, 30, and 60 minutes, and 2, 5, and 24 hours after addition of dextran sulfate plus calcium. It is evident from Table 4 that most of the cholesterol precipitates within the first few minutes, and that the precipitation is essentially complete within 60 minutes. As a matter of safety, however, we have usually kept our samples at 4° for 2 hours prior to centrifugation.

It is interesting to note that in the experiment reported in Table 4, no further precipitation of cholesterol occurred even for a period of as long as 24 hours. However, when the supernatant was kept at 4° for a period of 7 days, an additional amount of precipitate occurred. Of the 4.47 mg of cholesterol present in 3-ml serum, 0.50 mg precipitated in 2 hours, and an additional 0.10 mg precipitated in the next 6 days. The additional precipitate exhibited a cholesterol to phospholipid ratio of 0.43, which is very close to the cholesterol to phospholipid ratio of 0.47 of the original, dextran sulfate-soluble, α-lipoprotein. Moreover, paper electrophoresis of the redissolved precipitate showed the presence of α-, and a small amount of β-lipoprotein (Fig. 2, strip 6), as well as other proteins including albumin. Three milliliters of serum diluted with 0.42 ml of 0.9% NaCl was kept at 4° for the same length of time

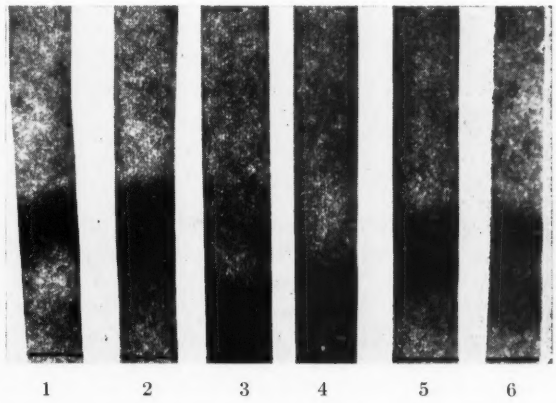


FIG. 2. Paper electrophoretogram of 50 λ of whole serum (strip 1), 50 λ of dextran sulfate supernatant from 3 ml of serum (strip 2), and 50 λ of dextran sulfate precipitate from 3 ml of serum redissolved in 1 ml of 0.9% NaCl and 0.1 ml K-oxalate (strip 3). The last strip shows the presence of a small amount of α-lipoprotein. Reprecipitation of this β-lipoprotein fraction with additional dextran sulfate and calcium chloride, and redissolution in 0.55 ml of NaCl, K-oxalate furnished a fraction essentially free of α-lipoprotein (strip 4). Strip 5 represents the supernatant 7 days after contact with dextran sulfate and $CaCl_2$ at 4°, and strip 6 the additional precipitate appearing between 2 hours and 7 days after dextran sulfate-$CaCl_2$ addition. Photographs were made with transmitted light.

as a control; this sample failed to show a precipitate. It appears, therefore, that after a prolonged period of time dextran sulfate, in the presence of calcium, will precipitate a small amount of proteins other than β-lipoproteins.

In order to obtain a more precise measure of the cholesterol to phospholipid ratio of β-lipoproteins of normal fasting male dogs, we precipitated the serum lipoproteins of eight additional dogs with dextran sulfate and calcium chloride. Afterward the precipitates were redissolved and subjected to ultracentrifugation at 105,000 × *g* at a density of 1.063 for 24 hours. By analyzing the top fraction in the centrifuge tubes, which corresponds to fraction [4] in Table 1, it was found that the cholesterol to phospholipid ratios of these beta, low density lipoprotein fractions were 0.85, 0.68, 0.55, 0.54, 0.66, 0.58, 0.78, and 0.55, with an average of 0.65 and a standard error of 0.013. This average value appears to be somewhat higher than the value of 0.53 reported by Havel *et al.* (8) for the fraction with a density between 1.019 and 1.063, but not quite as high as the value of 0.73 reported by Hillyard *et al.* (15) for the total low density fraction. As far as the relative proportions of α- or β-lipoprotein cholesterol are concerned, our eight male dogs showed an average content of 11% of β- and 89% of α-lipoprotein cholesterol. This proportion differs considerably from that reported by Castaigne and Amselem (2), who found 60% of the cholesterol in the β-lipoprotein fraction of dog serum by the dextran sulfate method. Hillyard *et al.* (15) reported 10%; Havel *et al.* (8), 9.5%; and Shafrir *et al.* (16), 11% of the cholesterol in the low density fraction.

DISCUSSION

Serum lipoproteins have been separated by ultracentrifugation, various forms of electrophoresis, and precipitation with sulfated polysaccharides. Although these procedures have been used by different investigators on human and animal sera, few comparative investigations of their respective values have been made. Particularly in animal sera in which the concentration of high density lipoproteins greatly exceeds that of the low density lipoproteins, it is useful to know which one of these procedures gives the best separation in the shortest possible time.

In our experiments it was shown that in dog serum, ultracentrifugation at a density of 1.063 failed to separate the high density and low density lipoproteins completely when such centrifugation was carried out according to the procedure of Havel *et al.* (8), which was designed for human serum. It was found that, in contrast to human serum, the high density lipoproteins of dog serum failed to settle in the bottom of the centrifuge tube, but instead exhibited a relatively uniform distribution throughout the tube. Only when centrifugation was carried out with smaller serum samples (5 ml), and for longer periods of time (24 hours), was a reasonably satisfactory separation of dog serum lipoproteins possible. Even then, however, one could judge only with difficulty the exact location of cutting the centrifuge tube for separation of high and low density fractions. This conclusion was reinforced by the centrifugation of dog serum prestained with Sudan black B, in which case an intense blue coloration was visible throughout the entire length of the centrifuge tube, although a slightly less colored region was found under the top layer in the tube containing 5 ml serum.

Among the variously available electrophoretic techniques, we chose that of paper electrophoresis. Although this technique does not lend itself easily to quantitative determination of α- and β-lipoproteins, it appears to be satisfactory for determining the purity of lipoprotein fractions obtained by other methods. The paper electrophoretic examination of the bottom layer of the ultracentrifuge tube after centrifugation for 24 hours at 105,000 × *g* disclosed the presence of two lipid-staining proteins, one with the mobility of α- and one with the mobility of β-lipoprotein. After dialysis of this solution for 24 hours against 0.9% NaCl, the two bands were still present and exhibited the normal mobilities of α- and β-globulins. The subnatant of density 1.063 was adjusted to a density of 1.21 and recentrifuged at 105,000 × *g* for 22 hours. The 7-ml bottom layer was dialyzed against 0.9% NaCl, concentrated to a small volume with Carbowax® (17), and an aliquot subjected to paper electrophoresis. This time lipid staining was observed only in a band corresponding to a mobility of β-globulin. Extraction of the lipids from the 1.21 bottom revealed the presence of phospholipid as the major lipid component. It appears, therefore, that in dog serum a lipid-containing β-globulin with a density greater than 1.21 is present.

The separation of lipoproteins with dextran sulfate appears to be the simplest procedure. However, this method too has its complications. It is not possible to obtain a really pure β-lipoprotein fraction. Although the second precipitate seems to be entirely free of α-lipoproteins, there is still present a little protein with the mobility of albumin. If one therefore is interested in the study of the protein portion of the lipoprotein, the dextran sulfate precipitated lipoprotein must be subjected to further purification.

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Influence of estradiol on apparent phosphatidyl choline synthesis in rats*

E. N. Bowser, W. J. Henderson, and H. J. Zimmerman

Radioisotope Service, Veterans Administration West Side Hospital, Chicago, Illinois; and the Department of Medicine, The Chicago Medical School, Chicago 8, Illinois

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SUMMARY

Oophorectomized Sprague-Dawley rats, 28 to 30 days old, were maintained for 31 days on either a normal diet or a high fat, low protein, low choline diet, with or without vitamin B_{12}. Twice weekly injections of sesame oil or estradiol in sesame oil were administered subcutaneously. The animals kept on the high fat, low choline diet showed severe fatty metamorphosis of the liver, a decreased hepatic lecithin concentration, and a twofold increase in the relative specific activity of lecithin. Estradiol inhibited fatty metamorphosis and increased the lecithin concentration slightly. The animals receiving a vitamin B_{12}-supplemented diet and estradiol showed the greatest lipotropic effect and an increased liver lecithin concentration. Estradiol alone, or combined with vitamin B_{12}, did not correct the apparent choline deficiency, as measued by the abnormally high rate of P^{32} incorporation into lecithin. The results suggest, therefore, that the prevention of fatty livers by estradiol depends on mechanisms other than those involved in the lipotropic properties of choline or vitamin B_{12}.

Previous studies by Gyorgy and Rose (1) and Emerson *et al.* (2) have shown that the administration of an estrogenic hormone prevents the development of fatty livers and cirrhosis in animals receiving a diet deficient in lipotropic agents. Studies in this laboratory have yielded similar results. Plagge *et al.* (3) found that the lipotropic effect of administered estradiol differed from that of methionine. Estradiol decreased the degree of fatty metamorphosis produced by a high fat, low choline diet, but did not increase growth. Methionine, on the other hand, not only inhibited the development of fatty livers, but also improved the growth rate. Nevertheless, the effect of estradiol on nutritional fatty livers might be related to an enhancement of the synthesis of choline or its precursors. The beneficial effect of vitamin B_{12} in experimental nutritional fatty livers has been related to the effect of this substance on the biosynthesis of methyl groups (4), and therefore vitamin B_{12} has been considered to possess choline-sparing action.

This study was designed to determine whether estradiol has a choline-sparing action (5). Choline availability was measured indirectly by the following changes in the liver: the severity of fatty metamorphosis, the concentration of lecithin, and the rate of P^{32} incorporation into lecithin of rats kept on a normal diet or a high fat, low choline diet, with and without added vitamin B_{12}.

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METHODS

Ninety castrated female Sprague-Dawley rats, weighing 75 to 95 g (28 to 30 days old), were divided into the following groups: I. a control group fed Rockland rat chow (C.D.); II. a group fed a high fat, low protein, low choline (HFLC) diet (3); and III. a group fed the above high fat, low protein, low choline diet but supplemented with 30 μg of vitamin B_{12} per kilogram of diet (HFLC + B_{12}). In each of the above groups approximately one-half were injected subcutaneously with sesame oil (0.1 ml) and the other half with estradiol propionate (0.1 mg in 0.1 ml sesame oil).

The rats were maintained for 31 days. Radioactive phosphate was injected intraperitoneally in a dosage of 1 μc/g of body weight 4 to 6 hours prior to sacrifice.

TABLE 1. EFFECT OF ESTRADIOL ON LIVER LIPID AND LECITHIN CONCENTRATION*

Treatment	No. of Rats	Avg. Initial Weight	Avg. Final Weight	Liver		
				Weight	Lipid	Lecithin
		g	*g*	*g*	*per cent*	*per cent*
Sesame						
C.D.	18	89.2 ± 5	212 ± 12	6.70 ± 0.85	4.47 ± 0.47	1.48 ± 0.23
HFLC	15	91.5 ± 6	79.6 ± 5	4.78 ± 1.05	24.1 ± 7.7	0.81 ± 0.19
HFLC + B_{12}	16	91.5 ± 4	74.6 ± 5	2.88 ± 1.00	20.2 ± 12.3	1.18 ± 0.24
Estradiol						
C.D.	16	86.2 ± 10	138 ± 6	5.06 ± 0.43	4.27 ± 0.78	1.40 ± 0.28
HFLC	12	82.9 ± 5	70.9 ± 5	3.12 ± 0.36	9.08 ± 3.2	1.05 ± 0.13
HFLC + B_{12}	12	83.2 ± 9	63.0 ± 7	2.42 ± 0.31	4.77 ± 1.43	1.30 ± 0.33

* Means ± standard deviation.

Liver tissue was extracted (6) and the total lipids weighed. The phospholipids were isolated (7) and lecithin separated (8). Total phosphorus (9) and inorganic phosphorus (10) were determined. Radioactivity was determined with a thin window G.M. tube to a counting error less than 2%. The relative specific activity (RSA) is defined as the specific activity of lecithin divided by the specific activity of inorganic phosphorus present in the tissue at the time of sacrifice. The RSA of lecithin at 4 and 6 hours following injection of inorganic P^{32} was determined as a measure of lecithin turnover and P^{32} incorporation.

RESULTS

The data on the lipid analysis of all groups studied are summarized in Tables 1 and 2. The livers removed from group I, oophorectomized rats, were found to have a normal lipid content even with the administration of estradiol. At autopsy, the livers from animals in group II, without estradiol, showed an approximately twofold enlargement relative to body weight, and exhibited severe fatty metamorphosis. The concentration of lecithin was found to be 33% less than that of the control rats. The RSA of lecithin was almost doubled for the 4- and 6-hour intervals studied.

TABLE 2. EFFECT OF ESTRADIOL ON THE RELATIVE SPECIFIC ACTIVITY OF LIVER LECITHIN AT 4 AND 6 HOURS AFTER INJECTION OF P^{32}*

Treatment	No. of Rats	4 Hours	No. of Rats	6 Hours
Sesame				
C.D.	10	0.64 ± 0.14	8	0.86 ± 0.30
HFLC	7	1.20 ± 0.08	8	1.54 ± 0.25
HFLC + B_{12}	8	1.15 ± 0.30	8	1.45 ± 0.50
Estradiol				
C.D.	9	0.67 ± 0.16	7	0.88 ± 0.36
HFLC	7	1.18 ± 0.21	5	1.38 ± 0.13
HFLC + B_{12}	5	1.13 ± 0.26	7	1.33 ± 0.32

* Means ± standard deviation.

In the estradiol-treated rats of group II, the per cent of liver lipid was 9.1 ± 0.3%. The lecithin was significantly increased ($p < 0.001$) when compared to the animals receiving HFLC diet without estradiol. The RSA value of this group, however, was abnormally high, as was that observed in animals receiving HFLC diet without estradiol.

The rats in group III, receiving HFLC + B_{12} diets, showed extreme variability in liver lipid content, which ranged from 5% to 35% with a mean of 20.2%. The lecithin concentration was similar to that of the control group.

The lipotropic effect of estradiol on rats maintained on HFLC + B_{12} was greater than that produced by the hormone in rats receiving the HFLC diet alone. The animals of group III had a normal hepatic lipid content and concentration of lecithin. The RSA values of lecithin were abnormally high in this group, as in the other groups fed the HFLC diet.

The animals in group II, estradiol-treated rats, ate less food per day than any other HFLC diet group studied (i.e., 3.8 ± 0.5 g per rat as compared to 5.3 ± 1 g).

DISCUSSION

The results of the present study indicate that the effects of a high fat, low protein, choline-deficient diet administered to the rat are similar to those in the dog, as reported recently by Di Luzio and Zilversmit (5). Both studies showed that in animals with fatty livers produced by this regimen there is an abnormally high rate of P^{32} incorporation into the phosphatidyl choline fraction, and a significant decrease in the hepatic concentration of lecithin. Artom (11) has suggested that livers of animals on diets deficient in protein and choline still have sufficient endogenous choline and

ethanolamine to show a compensatory increase in the rate of phospholipid synthesis.

Evidently the results of chronic administration of estradiol, unlike those results reported for chronic choline administration (5), produced a lipotropic effect without decreasing the high rate of phosphatide turnover. This is interpreted to mean that although estrogen appears to be lipotropic, it apparently does not influence the synthesis or availability of choline nor its methyl precursors sufficiently to correct the abnormally high rate of lecithin metabolism.

The lipotropic effect of estradiol was significantly increased by the presence of vitamin B_{12} supplement in the diet. This additive effect is analogous to that observed with the addition of small quantities of methionine to animals receiving estradiol and the HFLC diet (3).

Estradiol inhibits nutritional fatty metamorphosis as does choline (5) and vitamin B_{12} (12). The vitamin B_{12} administered to choline-deficient animals appears to reduce the requirement for choline through the synthesis of methyl groups (4). In this study the concentration of lecithin in livers was elevated toward normal by the presence of vitamin B_{12} in the diet without demonstrating a consistent effect on total lipids of the liver. The lipotropic action of vitamin B_{12} was dependent upon the fat composition of the diet, since it has been reported that on a high fat diet, vitamin B_{12} has no lipotropic action (12), whereas it is effective on a low fat diet (13). No significant effect was observed in this study using a diet containing 40% fat. It also has been demonstrated that the ability of the liver to store the vitamin is decreased in animals with nutritional liver disease (14).

Estradiol, vitamin B_{12}, and choline have been observed to increase the lecithin concentration of the liver in animals fed a HFLC diet (Table 3). Yet only choline reduces the apparent high rate of phospholipid synthesis characteristic of choline-deficient animals. In this study vitamin B_{12} and estradiol were equally effective in altering the RSA and were equally effective in increasing the lecithin content of the liver; however, B_{12} was far less effective than estradiol in "normalizing" liver lipid concentrations. Furthermore, estradiol apparently inhibited weight gain in animals consuming a normal and a HFLC diet, while choline and B_{12} have been reported to reverse the weight loss produced by diets deficient in protein and choline (15). The relationship of growth inhibition to lipotropism has been previously reviewed (16). These differences suggest that the mechanism of the lipotropic action of estradiol differs from that of choline and vitamin B_{12}.

TABLE 3. COMPARISON OF LIPOTROPIC EFFECTS OF ESTRADIOL, VITAMIN B_{12}, AND CHOLINE, BASED ON FOUR DIFFERENT PARAMETERS

Effects	Estradiol	B_{12}	Choline
Decreased total liver fat	+	+*	+
Increased lecithin content	+	+	+
Decreased abnormally high RSA	−	−	+
Reversed weight loss as produced by low protein diets†	−	+	+

* References 12, 13.
† Reference 16.

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Notes on Methodology

A method for the estimation of blood glycerides employing florisil*

DAVID H. BLANKENHORN, GEORGE ROUSER, and TRUCILLA J. WEIMER

University of Southern California School of Medicine, Los Angeles, California, and Department of Biochemistry, City of Hope Medical Center, Duarte, California

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» A rapid, reliable method for the direct determination of glycerides in human blood is required for many clinical studies. A suitable method is described in this report. A column of Florisil® (magnesium silicate) is employed to remove phospholipids from a lipid extract of plasma or serum, glycerides are then saponified, and glycerol determined by periodate oxidation. Methods based on similar principles have been developed by Carlson and Wadstrom (1), who have employed a column of silicic acid to separate glycerides from phospholipids, and by Van Handel and Zilversmit (2), and Cheng and Zilversmit (3), who have used zeolites to remove phospholipids by one-stage liquid-solid distribution. Radin *et al.* (4) have used Florisil for the recovery of cerebrosides free of phospholipid, while Carroll (5) has reported the separation of cholesterol, cholesterol esters, triglycerides, diglycerides, and monoglycerides on Florisil.

A glycerol standard solution is prepared from reagent grade glycerol. Specific gravity is determined by comparing the weight of glycerol and water delivered from a constant volume syringe. The water content of glycerol is then calculated (6) and a working standard solution (60 μg/ml) prepared. Solutions made with freshly opened glycerol and freshly distilled water remain unchanged for 18 months at 4° to 8°, and 2 months at room temperature if protected from contaminating molds.

Four per cent alcoholic potassium hydroxide solution (95% ethanol) is prepared each week, protected from air and light, and discarded when brown or turbid.

Chromotropic acid solution is prepared by dissolving 1 g of 4,5-dihydroxy-2,7-naphthalene disulfonic acid disodium salt (practical grade, Distillation Products, Rochester, New York) in 100 ml of water. The solution is filtered and made to 500 ml with 11 M reagent grade sulfuric acid. Chromotropic acid is stored in brown, glass-stoppered reagent bottles. Different lots usually produce blanks having different absorbencies that increase slowly as solutions age. Freshly made chromotropic acid should not have an absorbency greater than 0.045 when read at 570 mμ in a 1-cm cell against distilled water.

Ten normal sulfuric acid, 0.1 M sodium periodate solution, and 1.0 M sodium arsenite solution are prepared as directed by Lambert and Neish (7).

All solvents are reagent grade and all are redistilled in glass apparatus except for anhydrous reagent grade diethyl ether (peroxide-free, purchased in cans).

The water content of Florisil[1] (60 to 100 mesh) is determined by bringing an aliquot to constant weight at 105°. Enough water is then added as a fine spray from a syringe fitted with a No. 25 needle while shaking Florisil in an Erlenmeyer flask to give 40 mg of water per gram of adsorbent. This water content was selected after trial with anhydrous Florisil and adsorbent containing 20, 40, and 70 mg/g indicated that 40 mg/g was the minimum water content for essentially complete retention of lipid phosphorus with rapid and complete elution of glycerides.

Acetone-alcohol 1/1 (v/v) is used for extraction of lipids; the method has been found to yield similar results with chloroform-methanol 2/1 (v/v), and ethanol-ether 3/1 (v/v) extracts. One milliliter of serum or plasma is sprayed by forceful ejection from a calibrated syringe through a No. 25 needle into 25 ml of acetone-alcohol in extraction tubes, which are then closed with polyethylene screw caps (Belart Division of A. S. Aloe, Los Angeles, California; not attacked by acetone-alcohol). The tubes are shaken mechanically for 10 min-

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[1] Floridin Co., Tallahassee, Fla.

utes and then centrifuged briefly to pack precipitated protein. Six milliliter aliquots are withdrawn, dried under a stream of air at 55°, and taken up in 3 ml of a mixture of petroleum ether (60°–75°) and diethyl ether 95/5 (v/v).

Funnel tubes 300 x 4 mm (I.D.) are used for columns (Kimble, No. 46185, Owens-Illinois Glass Co., Owens, Illinois). These are plugged with surgical cotton previously extracted twice with chloroform. Florisil (700 to 800 mg) is packed loosely to give a height of approximately 125 mm. The petroleum ether-diethyl ether solution of lipid is then transferred to the bed and a small volume (1 to 2 ml) of the same solvent is used to effect quantitative transfer of the charge onto the column. Next, 8 ml of chloroform-acetone-water 99/99/2 (v/v) is added and collected with the charging solution in a 25-ml volumetric flask. Twelve columns can be run simultaneously. Two columns are not charged; petroleum ether-diethyl ether and chloroform-acetone-water solutions are passed through each into two volumetric flasks which are later used for the glycerol standard and reagent blank.

The effluents in volumetric flasks are taken to dryness under a stream of air at 55° for 15 minutes (it is important to remove the solvent completely). Alcoholic KOH (0.25 ml) and 95% ethanol (2.5 ml) are added. Flasks are gently shaken and placed in a 55° water bath for 30 minutes. Next, 0.25 ml of 10 N sulfuric acid and 2.5 ml of water are added. One milliliter of 0.1 M sodium periodate solution is added, and exactly 5 minutes later 1 ml of 1 M sodium arsenite solution is added. Iodine liberated in the reaction produces a yellow or brown color which fades rapidly, but may not disappear completely, until flasks are made to volume with water. After solutions are made to 25 ml volume, 2-ml aliquots are withdrawn, mixed with 10 ml of chromotropic acid solution, placed in a 100° bath in the dark for 30 minutes, removed, and read at 570 mμ. A blank solution and a glycerol standard are carried through saponification and periodate oxidation in flasks previously set aside. Repeat samples for color development can be drawn up to 36 hours later and aliquots that vary in size from 1 to 3 ml can be reacted with 10 ml of chromotropic acid.

TABLE 1. TRIPLICATE GLYCERIDE-GLYCEROL DETERMINATIONS ON 10 SERUM EXTRACTS

Serum Extracts	Assay Number 1	2	3
	mg/100 ml		
1	10.5	11.9	10.7
2	17.5	16.6	16.7
3	15.6	17.2	17.7
4	10.9	11.7	11.1
5	0.3	1.5	1.1
6	11.1	11.9	11.1
7	13.9	14.3	14.3
8	16.6	18.0	17.6
9	10.8	10.0	9.8
10	14.8	14.9	15.1

Triplicate assays performed on 10 serum extracts are shown in Table 1. Calculated from these data, the standard deviation of a single determination is 0.6 mg glyceride-glycerol per 100 ml serum. The average glyceride-glycerol content is 12.8 mg/100 ml, a value in satisfactory agreement with those previously reported by others (1, 8).

The removal of phospholipid from 16 serum extracts containing 12.3 to 29.3 μg phosphorus (average 20.1 μg) was studied. No detectable phosphorus could be found in 10 extracts after passage through Florisil. From 0.01 to 0.13 μg (average 0.07 μg) of apparent phosphorus was found in six column effluents. The apparent amount in the eluates was not related to the amount of phosphorus charged, and was at the lower limit of the accurate range of the sensitive method employed (9).

Recovery of added glycerides was studied with six serum extracts. One milliliter of serum was extracted, as described above, and 1 ml each of mono-, di-, and triglyceride solutions containing 5 μg of glycerol per ml was added to 3 ml of the extract. The total amount of glycerol in each mixture was approximately that expected from 6 ml of serum extract. Duplicate recoveries averaged 101%. Similar results were obtained with the extract from serum 5 (Table 1), indicating that the low glyceride-glycerol level found without additions for this sample was not in error.

Florisil appears to have several advantages over previous substances used to adsorb phospholipids from serum extracts. The ease with which Florisil can be handled constitutes a major advantage over silicic acid. Minimal preparation of Florisil yields a reproducible adsorbent readily packed into long and narrow columns to give increased resolution with rapid, reproducible flow rates. Quantitative transfer of the charge is readily performed with funnel tubes, and after the charge is adsorbed, the entire volume of wash solution can be added so that columns do not require further attention. These columns have been found to be very suitable for multiple, precise assays.

The advantage of Florisil over the zeolite Duocil is that it allows recovery of all glyceride classes without passing significant amounts of phosphorus. In our hands, only a variable proportion (1% to 40%) of monopalmitin added to human serum extracts was recovered from Duocil activated as described by Van

Handel and Zilversmit (2). Eluates from Duocil prepared in this manner also contained significant amounts of phosphorus (0.6% to 6.0% of the charge). Because Carlson and Wadstrom (10), Hirsch and Ahrens (11), and Mead and Fillerup (12) have presented evidence that partial glycerides can occur in human blood, it seems important to be certain that all glyceride classes are included when blood is assayed for glyceride-glycerol. In addition, when sera with extremely low glyceride-glycerol content, such as serum 5 (Table 1), are assayed, the presence of small amounts of phospholipid may introduce major analytical error. Florisil columns as described here have been found to pass so little phospholipid that the error introduced into glyceride-glycerol determinations will not exceed 0.12 mg/100 ml serum, while all glyceride classes are recovered quantitatively.

In the present method periodate oxidation for glycerol determination is performed by the method of Lambert and Neish (7), as modifications for the determination of smaller amounts of glycerol have not been required. The original method appears to be more reliable for routine applications. The present method differs from other direct blood glyceride-glycerol methods in that a glycerol standard solution is used. Glycerol can be readily obtained in pure form and standardized by weight, thus avoiding uncertainty that may be encountered in the use of triglyceride standards.

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A micro dibromide procedure for purifying radioactive cholesterol*

JON J. KABARA and JAMES T. MCLAUGHLIN

Biochemistry Section, Department of Chemistry, University of Detroit, and Mount Carmel Mercy Hospital, Detroit 21, Michigan

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» The interpretation of isotope experiments on cholesterol metabolism will be subject to uncertainty until the contribution of related companion sterols can be evaluated. The error in assuming homogeneity of the initially isolated sterol can be considerable (1 to 4). For the further purification of the isolated sterol, a micro dibromide procedure was needed. For the manipulation of 1-mg amounts of cholesterol the usual macromethods (2, 5) cannot be used. The scaled-down quantities of glacial acetic acid and cold methanol of the macromethod still dissolve small amounts of dibromide. Consequently, the macromethod was modified, and the resulting micromethod previously reported (6) is presented in detail.

MATERIALS AND METHODS

The chemicals used in these experiments were C.P. or reagent grade and required no further purification. However, the condition of the zinc granules was found to be critical. Upon standing, the metal becomes coated with carbonate, which seriously impairs its reactivity to acetic acid. The zinc, therefore, was washed with concentrated HCl followed by distilled water until evolution of hydrogen stopped. The metal was then used immediately.

Cleavage of the Cholesterol-Glycoside Complex. Cholesterol isolated from biological material was recovered by precipitation with digitonin (3, 4) or with the new glycoside tomatine (7). Before bromination of cholesterol it was necessary to cleave this insoluble complex. The digitonide was readily cleaved by the pyridine method of Schoenheimer and Dam (8). The tomatinide can be split by dissolving it in dimethylformamide as reported by Schulz and Sander (9). In our hands the latter method was not reliable and the following method of hydrolysis was introduced.

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The cholesterol tomatine complex representing 1 mg of cholesterol was dissolved in 1.0 ml of acetic acid and treated with an equal volume of 40% NaOH (w/v). The solution was thoroughly mixed and heated on a water bath (55° to 60°) for 30 minutes. The solution was then extracted three times with 3 to 4 ml portions of diethyl ether. The ether fractions were combined and carefully evaporated (35° to 40°) to 3 to 4 ml, and the excess acetic acid in the ether was neutralized with 10% sodium hydroxide solution using phenolphthalein as an indicator. The ether layer containing the free sterol was separated from the slightly basic aqueous phase and concentrated to 1 ml.

Bromination of Micro Quantities of Cholesterol. To the above ether solution, liquid bromine was added until it assumed a slight orange color. An excess of bromine interferes with the formation and isolation of the dibromide derivative. After 1 hour at room temperature, 3 ml of acetic acid was added, and the tubes were immersed in an ice bath. When the solution was thoroughly chilled, ice water was added until the first appearance of a precipitate. Crystallization was then allowed to continue at room temperature for 1 or 2 hours, or preferably overnight.

Debromination of 5α,6β-Dibromo Cholestane-3β-ol. The solid dibromide derivative was separated from the solution by centrifugation, washed twice with 3 ml of distilled water, and dissolved with 3.0 ml glacial acetic acid. After addition of 10 granules of zinc, the reaction mixture was heated on a water bath (60°) for 30 to 60 minutes and then cooled to room temperature. The solution was separated from the unreacted zinc by decantation and the granules washed twice with 1-ml portions of acetic acid. The filtrate and combined washings were neutralized to phenolphthalein with 10% sodium hydroxide, and then extracted with several portions of ether. The ether solution was evaporated to near dryness, and the purified cholesterol dissolved in 2 to 3 ml of acetone-alcohol-ether 4/4/1 (v/v). In order to avoid quenching by residual halogen in our liquid scintillation counting system, the purified cholesterol was reisolated and assayed as the tomatinide as previously described (7). Starting with a 1.0 mg sample, 30% to 4% yields were reproducibly recovered. With quantities greater than 1.0 mg, the percentage yields were correspondingly higher.

DISCUSSION

In order to brominate cholesterol isolated either as the digitonide or tomatinide, it was necessary to split the complex by using alkaline hydrolysis. The concentration of alkali and the conditions under which cleavage was brought about were sufficiently mild so as not to cause noticeable degradation, as demonstrated by recovery of the sample in high yields. The amount of bromine used for the halogenation was critical. If the solution became too dark an orange, it was not possible to isolate the dibromide derivative. For precipitation of the dibromide derivative, it was imperative that cold water be added dropwise; too little as well as too much water resulted in poor yields. Under these conditions of water addition, nonbrominated cholesterol was soluble and could not be precipitated. To eliminate the possibility that nonbrominated cholesterol had co-precipitated with the dibromide derivative, the precipitated derivative was isolated, redissolved in acetic acid, and the presence of free sterol tested with the Liebermann-Burchard reagent. Failure to develop color under these conditions verified that the precipitated compound contained less than 30 mg cholesterol.

The purification of radioactive cholesterol via the dibromide is considered the best procedure for eliminating higher counting companions of the sterol (1). In agreement with Schwenk and Werthessen, a single bromination and reconversion to cholesterol was usually sufficient to establish radiochemical purity of the isolated sterol.

Table 1 represents data typical of studies on humans.

TABLE 1. RADIOCHEMICAL PURITY OF CHOLESTEROL ISOLATED FROM HUMAN PLASMA AS THE DIGITONIDE

Time After I.V. Acetate 1-C^{14}*	Initial Specific Activity	Macro† Dibromide	Micro‡ Dibromide
Minutes	*mc/g*	*mc/g*	*mc/g*
10	0.057	0.026	0.030
20	0.120	0.103	0.105
30	0.241	0.235	0.227
60	0.345	0.340	0.343

* 100 μc.
† >100 mg (see Ref. 1).
‡ <10 mg.

The macro dibromide method (1) and our micromethod are in good agreement. The use of the micromethod for the purification of H^3-labeled cholesterol isolated as the tomatinide is reported elsewhere (7)

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Lipid Distribution Program Sponsored by National Institutes of Health

Through a co-operative project of the Division of Research Grants and the National Heart Institute, National Institutes of Health, ampoules containing 200 mg of mixtures of pure fatty acid methyl esters will soon be available for distribution to qualified investigators in whose research programs such materials are needed. These samples should be useful in the calibration and standardization of analytical techniques requiring only small amounts of lipids, and additional literature will be made available to assist research workers who wish to apply them to gas-liquid chromatography.

Investigators desiring these samples should describe briefly the research purposes for which they are to be used. The committee advising on this distribution program will attempt to fill all deserving requests, but with the aim also of assuring broad distribution to areas where they may be needed. Letters should be addressed in duplicate to Dr. William H. Goldwater, Executive Secretary, Metabolism Study Section, Division of Research Grants, National Institutes of Health, Bethesda 14, Maryland.

J. Lipid Research, July, 1961
Volume 2, Number 3

New Methods

» New Methods is a listing of pertinent references to analytical lipid methods published recently. It is compiled by David Kritchevsky, H. A. I. Newman, and Norman S. Radin. (References opened with No. 1 in the January, 1961, issue of the JOURNAL OF LIPID RESEARCH.)

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INSTRUCTIONS TO AUTHORS

Scope: The JOURNAL OF LIPID RESEARCH, a quarterly, will publish original articles dealing with the chemistry, biochemistry, enzymology, histochemistry, and physiology of the lipids. The editors will favorably consider significant contributions in the field of lipid methodology and will encourage publication of sufficient details so that the methods can be reproduced. Clinical observations and nutritional data will be welcomed if they offer contributions in the above fields. In addition to original articles, the JOURNAL will contain review articles and a section, "Notes on Methodology," dealing primarily with minor improvements in existing methodology. Notes on Methodology will not generally require a summary or subdivision into sections. A listing of new lipid methods published in other journals will also be provided. Although articles will be published in English only, it is hoped that the JOURNAL will become a vehicle for international communication.

Preparation of manuscripts: All manuscripts, including tables and figures, should be submitted in duplicate. Manuscripts should be typewritten, double-spaced, with minimum page margins of one inch. The first page should be a title page, consisting of: title; author(s); institution from which the paper is submitted, including the complete postal address(es) for mailing of proofs and reprint requests; and an abbreviated title for a running head (not to exceed 60 characters, counting the spaces between words). A notation should be made when the manuscript is intended for the Notes on Methodology section. Footnotes on the title page and those to tables should be indicated by non-numerical symbols (asterisk, dagger, double dagger, etc.), but in the text should be numbered consecutively and typed on a separate sheet.

Manuscripts should be written in clear, grammatical English. Unusual abbreviations should be used as little as possible, and must always be initially defined. Each article should be preceded by a Summary, not to exceed 200 words. Whenever possible, Methods, Results, and Discussion should be set forth in separate sections with appropriate headings. These headings should be in capital letters, centered on the page; subheadings should be in lower case and underlined for italicizing in print. All drawings and illustrations should be submitted in the form of glossy prints. Each should be identified on the back by figure number and author's name, using a *soft pencil* to avoid marring the print.

Tables should be typed on separate sheets and numbered with Arabic figures. All tables should be self-explanatory. Draw graphs and diagrams in *black* ink.

For the terminology of lipid enzymes the JOURNAL will follow the recommendations published in "Nomenclature of enzymes of fatty acid metabolism" in *Biochemical Problems of Lipids*, edited by G. Popják and E. Le Breton, London, Butterworths Scientific Publications, 1956, p. 246.

For the terminology of serum lipoproteins the JOURNAL will follow the recommendations published in *Circulation Research* **4**: 129, 1956.

The following are the accepted abbreviations for units of measurement:

Units of Mass:	
kilogram	kg
gram	g
milligram	mg
microgram	μg
millimole	mmole (not mM)
micromole	μmole (not μM)
Units of Concentration:	
molar (mole per liter)	M
millimolar	mM
micromolar	μM
Units of Length, Area, Volume, etc.:	
meter	m
centimeter	cm
millimeter	mm
millimicron	mμ
square centimeter	cm^2
liter	liter
milliliter	ml
cubic centimeter	cc or cm^3
microliter	μl
counts per minute	cpm
counts per second	cps
millicurie	mc
microcurie	μc
temperature (Centigrade only)	°

The expression mg% should not be used, but should be written as mg/100 ml. For chemical nomenclature the JOURNAL will follow the conventions of *Chemical Abstracts*. The superscript ® should follow all trade names. When necessary for identification of other specific materials, use a footnote, including name and location of manufacturer. Use the per cent sign (%) with figures.

References should appear in numerical order throughout the text. Bibliographic references must be typewritten, double-spaced, on a separate sheet, using the following style:

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FOR BOOKS: Author's last name, initials. *Title of Book*. City, Publishing House, year, page number.

FOR ARTICLES IN BOOKS: Author's last name, initials. In *Title of Book*, initials and name of editor(s), City, Publishing House, year, volume, page number.

For examples of these styles, see issues of the JOURNAL.

Mention of "unpublished experiments," "personal communications," "papers submitted," etc., should be made as footnotes and not included in the references. References to papers which have been *accepted* for publication but not yet printed, are cited in the bibliography as are other references, followed by the words "in press."

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